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THE CARDIAC OUTPUT IN MAN: STUDIES WITH THE LOW FREQUENCY, CRITICALLY-DAMPED BALLISTOCARDIOGRAPH, AND THE METHOD OF RIGHT ATRIAL CATHETERIZATION [1]

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Although the determination of cardiac output from the ballistic recoil of the body with each heart beat has been used repeatedly during the past 40 years, it has not been entirely satisfactory for general clinical use. Recently a survey of the problem has been made by Nickerson and Curtis (1), and as a result of this investigation they have recommended certain conditions to be satisfied in the construction of the ballistic system. Since a simple and reliable clinical method for the determination of cardiac output is of considerable importance, we have undertaken to test the type of ballistocardiograph described by Nickerson and Curtis. Studies have been made measuring the cardiac output by the ballistocardiograph, and by the method of right atrial catheterization utilizing the Fick principle.

APPARATUS

The ballistocardiograph used in this study differs from the instrument described by Starr *et al.* (2) in two main respects: (*1*) the undamped natural frequency of the bed is low; (*2*) the system is critically damped. The frequency of our bed is 1.5 cycles per second, whereas that of Starr was much more rapid. The bed described here is damped so that when it is deflected from its resting position, it returns to the original position with a minimum of overshooting. This is "critical damping" in the terminology of the physicists.

The apparatus used in this study differs from that of Nickerson and Curtis (1) only in that it was constructed for mounting on the fluoroscopic table upon which the right atrial catheterizations were performed. This arrangement permitted the almost simultaneous determination of the cardiac output by the technique of right atrial catheterization and by the ballistic method.

The ballistic bed consisted of a flat wooden table-top mounted on 4 flat strips of spring steel (Figure 1), so that movement in the longitudinal direction only was possible. The weight of the moving parts of the bed was about 75 pounds; the actual amount was not at all important, since variations in it were accounted for in the calibration to be described. Observations of the effect of increasing the weight of the moving part of the bed have led us to believe that it is more important that the bed be rigid than that it be of light weight. The effective length of the 4 springs upon which the bed was mounted was adjustable by means of clamps (Figure 1), so that the undamped frequency of the ballistic system could be brought to the same value (1.5 cycles per second) whatever load was placed on the bed. (The same result could be attained by keeping the spring lengths constant and supplementing the patient's weight by dead weights, thus maintaining the total load and hence the undamped frequency constant.) It should be noted that when calibrating the bed to its proper frequency for various loads, the damping bellows, emptied of oil, should be connected in the system so as to contribute its part to the restoring forces determining the movement of the bed. It is also important that the sylphon bellows be as flexible as possible in order to add but little to the restoring forces. The most satisfactory bellows requires a force of not more than 0.5 pound to compress it about 0.100 inch. The proper position of the clamps for each load was marked on a scale graduated in steps of 5 kgm., so that once calibrated, the setting of the ballistic bed to the nearest 5 kgm. for a subject of any weight was a rapid and simple procedure.

After the frequency calibration had been completed, the damper bellows, tube and reservoir (Figure 1) were filled with oil, care being taken to exclude air bubbles from the system. The position of the damping rod to produce critical damping for each load was then determined. The condition of critical damping can be practically considered as that situation where the bed on release from a displaced position moves back to its resting place with a minimum detectable overshoot. The calibration was made for a wide range of bed loads and over an oil temperature range corresponding to the room temperatures customarily found. When these calibrations were completed it was sufficient to know the oil temperature and the patient's weight in order to set the damping rod at the proper value.

The movements of the bed were recorded optically on

[1] The work described in this paper was done under contracts, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University and also between the Office of Scientific Research and Development and Emory University School of Medicine.

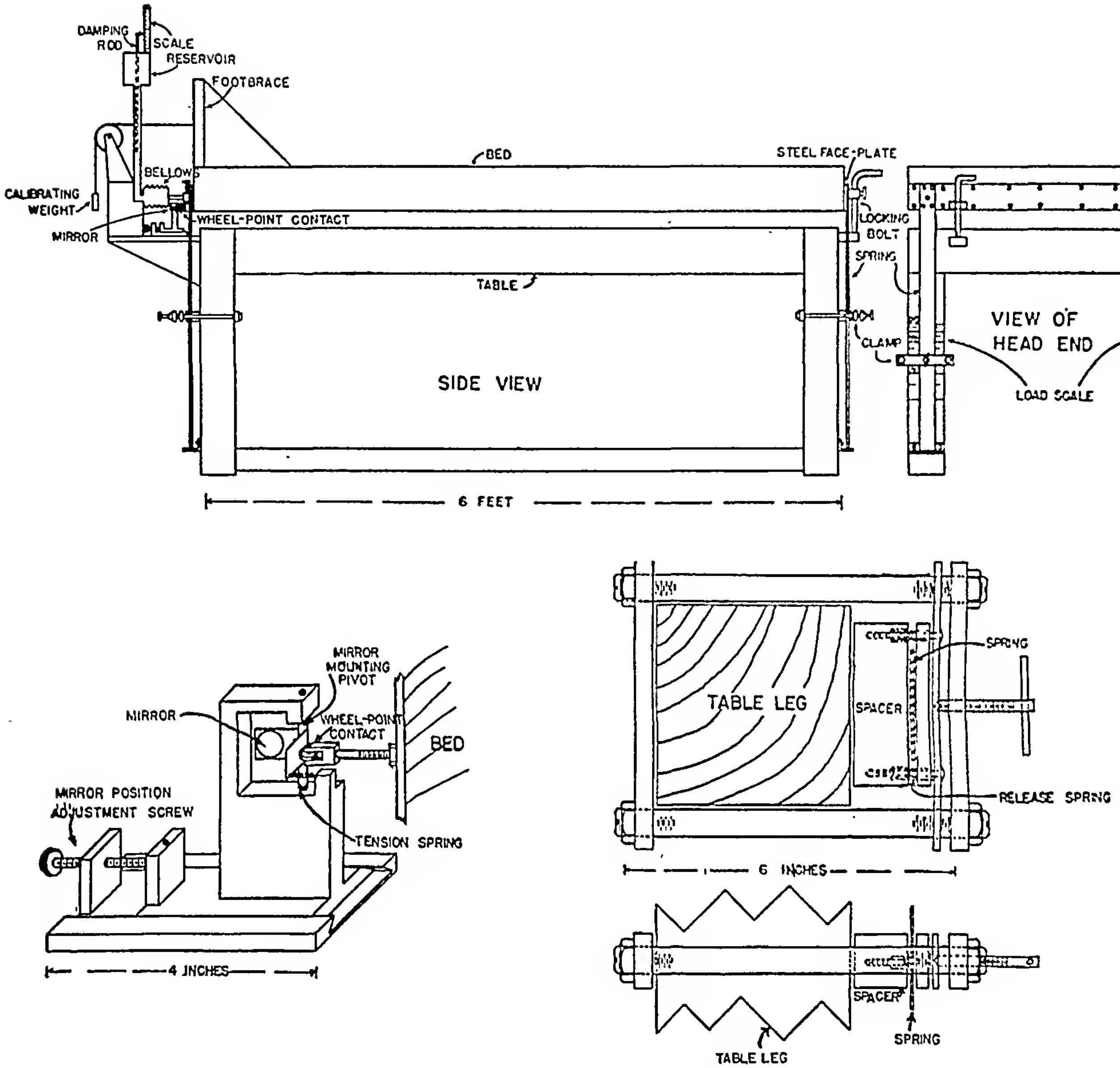

FIG. 1. DETAILS OF THE CONSTRUCTION OF THE BALLISTIC BED AS MOUNTED ON A TABLE

The figure shows the flat springs on which the bed is supported, the spring clamps, the locking bolt damping system, the pickup mirror mounting, and wheel-point contact. The spring clamp and mirror sy are shown in considerable detail.

photographic paper. A light beam was reflected from a small pivoted concave mirror, which was held by means of a small spring against a frictionless wheel point moving with the ballistic bed (Figure 1). The movements of the reflected light were recorded by a camera similar to that used in an electrocardiographic apparatus.

METHOD

The subject was placed on his back on the ballistocardiograph with the soles of the feet tight against the upright foot-board, and the breath held in mid-position while the tracing was being taken. Figure 2 shows a typical tracing, with the headward deflections recorded in the upward direction, and the footward deflections in the downward direction. The initial major footward tion (I) resulting from the recoil due largely to tion of blood by the heart, and the initial maj

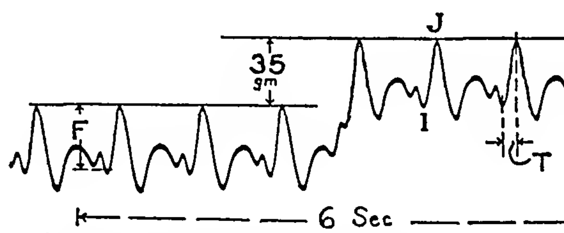

FIG. 2. A CHARACTERISTIC BALLISTOCARDIOGRAPH

This figure demonstrates the method of calibrat indicates the measurements to be taken from the

ward deflection (J), resulting in the main from the slowing of blood in its headward course and from its turning around the aortic arch, are the most important landmarks in the tracing. A force of 35 grams was applied to the system during a portion of the tracing by weights attached to the foot of the ballistic bed and hanging over a light frictionless pulley. This offered a means of calibrating the bed while it was under the actual conditions of use, by showing how far the base line was displaced by a standard force. Knowing the displacement caused by the standard force, the displacement of the major deflection can be converted to the force involved (F) by measuring the distance between the peak of the first major footward deflection (I) and the peak of the first major headward deflection (J) and making a simple proportionality calculation. Two other measurements were required from the tracing: (*1*) the heart rate, and (*2*) the time interval in seconds (T) between the peak of the first major footward movement (I) and the peak of the first major headward movement (J).

The formula used for computing the cardiac output is slightly modified from the basic equations discussed in an earlier paper (3). The stroke volume is given by:

$$SV = \frac{5.02\, F\, P}{T\, L}\ \text{ml.},$$

and the cardiac output equals the stroke volume multiplied by the heart rate, where F and T have been defined above and, along with the heart rate, are obtained from the ballistic record. The quantity L is the height of the subject in centimeters, and 5.02 is a constant determined to adjust the ballistic results to a best fit with the catheter data. The factor P over most of the range of blood pressures, equals the square root of the arithmetic mean of the systolic and the diastolic pressures. However, at low pressures the results computed using $\sqrt{P_a}$ in the formula were somewhat too large, since with the correspondingly small stroke volumes, the momentum of the center of gravity of the heart muscle provided an appreciable part of the impact. For this reason, a table of P values (Table I) has been provided which gives a better fit for the data on normal subjects. As seen in Table I, the values of P differed only from $\sqrt{P_a}$ at low pressures.

The methods used in the cardiac output determination by the catheter technique have been described elsewhere (4). Samples of mixed venous blood were obtained by passing a flexible radiopaque catheter through the venous system into the right side of the heart. Arterial blood was obtained from an inlying needle in the femoral artery. Oxygen consumption was measured by collecting a 2- or 3-minute sample of expired air and analyzing its contents. From these data, utilizing the Fick principle, the cardiac output was calculated as follows:

$$\text{cardiac output} = \frac{\text{oxygen consumption}}{\text{arteriovenous difference}}.$$

Because the output of the heart varies with body size, we have facilitated comparison between individuals by expressing the output in terms of cardiac index. This is the cardiac output in liters per minute per square meter of body surface.

TABLE I

P_a	$\sqrt{P_a}$	P	P_a	$\sqrt{P_a}$	P
50	7.1	5.0	100	10.0	10.0
52	7.2	5.3	102	10.1	10.1
54	7.3	5.6	104	10.2	10.2
56	7.5	5.8	106	10.3	10.3
58	7.6	6.1	108	10.4	10.4
60	7.7	6.4	110	10.5	10.5
62	7.9	6.7	112	10.6	10.6
64	8.0	7.0	114	10.7	10.7
66	8.1	7.2	116	10.8	10.8
68	8.2	7.5	118	10.9	10.9
70	8.4	7.8	120	11.0	11.0
72	8.5	8.1	122	11.0	11.0
74	8.6	8.3	124	11.1	11.1
76	8.7	8.5	126	11.2	11.2
78	8.8	8.7	128	11.3	11.3
80	8.9	8.9	130	11.4	11.4
82	9.1	9.1	132	11.5	11.5
84	9.2	9.2	134	11.6	11.6
86	9.3	9.3	136	11.7	11.7
88	9.4	9.4	138	11.7	11.7
90	9.5	9.5	140	11.8	11.8
92	9.6	9.6	142	11.9	11.9
94	9.7	9.7	144	12.0	12.0
96	9.8	9.8			
98	9.9	9.9			

In obtaining the data reported here, all subjects, except the patients in shock, were studied in the morning 15 hours after the last meal. The catheter was passed and the arterial needle inserted. After a variable period of time, during which the subject was able to relax and reach a relatively stable state, the observations were made. At first, ballistic tracings were obtained both before and after the samples were taken for the Fick output. Later, because these tracings varied so little, they were obtained only either immediately before or after the Fick output determinations.

RESULTS

Eighty-one cardiac output determinations by the catheter technique and almost simultaneous ballistic tracings were made on 58 individuals. The data obtained on the first 50 of these determinations on subjects without heart disease or myxedema were used to evaluate empirically the constants in the stroke volume equation. The stroke volume constant 5.02, and the values of the pressure factor P given in Table I are the results of these determinations. Once determined, these constants, which gave the best fit of the ballistic and

the catheter data, were used throughout this study. This method of computing the cardiac output from the ballistic records does not invalidate a comparative study of the results of the 2 methods. Actually, it tests the success of the formula in fitting the ballistic data to that obtained by the application of the Fick principle. It should be remembered, however, that because of the empirical adjustment, the cardiac output by the ballistic method is dependent on the values obtained by the

TABLE II

Subject	Sex	Age	Ht.	Wt.	S.A.	Fick CI	Pressure P_s	Pressure P_d	F.	T.	HR	BCG CI
		yrs.		*lb.*	*sq. m.*	*l. per min. per sq. m.*	*mm. Hg*		*grams*	*sec.*	*per min.*	*l. per min. per sq. m.*
L.A.	M	15	5′7″	131	1.68	2.3	89	58	26.0	.128	81	2.4
L.A.	M	15	5′7″	131	1.68	3.5	107	61	35.0	.124	75	3.4
L.A.	M	15	5′7″	131	1.68	4.3	124	65	45.0	.126	75	4.6
E.J.	F	30	4′11″	90	1.31	2.1	103	59	11.6	.140	103	2.0
E.J.	F	30	4′11″	90	1.31	2.7	111	59	19.8	.134	97	3.4
E.J.	F	30	4′11″	90	1.31	4.0	116	62	26.6	.140	97	4.4
S.M.	M	39	6′2″	150	1.91	2.3	82	49	32.0	.124	107	2.8
S.M.	M	39	6′2″	150	1.91	4.0	93	49	45.0	.128	94	3.7
S.M.	M	39	6′2″	150	1.91	5.6	102	49	65.0	.124	90	5.6
S.M.	M	39	6′2″	150	1.91	5.9	102	50	77.0	.128	90	6.4
W.T.	M	52	5′7″	167	1.87	1.5	71	42	40.3	.148	70	1.8
W.T.	M	52	5′7″	167	1.87	2.9	122	63	34.1	.154	82	2.8
M.Z.	F	22	5′1″	110	1.46	2.2	86	47	22.3	.123	100	3.0
M.Z.	F	22	5′1″	110	1.46	4.8	130	75	34.0	.130	96	5.6
P.D.	M	34	5′7″	130	1.67	3.1	76	46	45.0	.168	78	2.5
N.A.	M	33	5′10″	135	1.75	2.9	112*	60*	36.0	.138	62	2.4
N.A.	M	33	5′10″	135	1.75	2.7	120*	63*	47.0	.138	54	2.8
N.A.	M	33	5′10″	135	1.75	2.6	120*	63*	47.0	.135	57	3.1
J.B.	M	30	5′11″	189	2.04	3.3	133	75	55.0	.144	60	3.2
J.B.	M	30	5′11″	189	2.04	2.8	155	86	48.0	.133	62	3.3
J.B.	M	36	5′9″	189	2.00	3.6	125	75	40.0	.137	78	3.3
J.B.	M	36	5′9″	189	2.00	4.4	155	79	60.0	.141	60	4.0
L.L.	M	16	5′8″	139	1.73	2.5	119	68	46.5	.137	51	2.8
L.L.	M	16	5′8″	139	1.73	3.3	142	78	35.0	.124	60	3.0
L.L.	M	16	5′8″	139	1.73	3.1	142	75	37.0	.129	52	2.6
A.R.	M	40	6′1″	146	1.87	3.6	179	106	35.9	.162	71	2.7
A.R.	M	40	6′1″	146	1.87	3.4	179	106	32.8	.158	81	2.9
J.S.	M	30	5′8″	142	1.75	2.6	117	65	47.0	.161	54	2.5
J.S.	M	30	5′8″	142	1.75	3.6	123	71	47.0	.147	60	3.1
W.W.	M	57	5′6″	145	1.73	3.1	163	86	33.0	.120	64	3.4
W.W.	M	57	5′6″	145	1.73	5.1	166	84	42.0	.120	65	4.4
W.W.	M	57	5′6″	145	1.73	5.2	150	77	40.0	.120	60	3.7
Y.A.	M	16	5′5″	116	1.56	2.5	110	60	43.2	.166	65	3.0
W.B.	M	17	5′4″	105	1.48	4.3	152	84	32.0	.120	78	4.7
O.F.	M	31	5′11″	155	1.87	3.0	115	66	50.3	.146	62	3.0
E.G.	M	29	5′6″	121	1.60	3.1	121	63	31.0	.148	60	2.3
M.J.	F	21	5′4″	104	1.46	4.6	110	61	32.0	.120	78	4.1
V.M.	M	18	5′6″	153	1.77	4.5	127	64	70.0	.176	65	4.3
J.W.	M	42	5′6″	135	1.68	2.8	114	58	34.0	.168	75	2.5
C.C.	M	36	6′1″	166	1.97	3.7	170	94	63.0	.142	73	5.1
J.E.	M	48	5′11″	148	1.84	4.6	163	83	34.6	.133	98	4.3
B.E.	F	22	5′4″	129	1.61	5.5	152	68	52.0	.131	75	6.0
R.C.	M	50	5′3″	126	1.62	4.6	133	57	45.8	.117	71	5.3
R.C.	M	50	5′3″	126	1.62	3.0	137	60	28.4	.118	43	2.0
I.M.	F	22	5′4″	94	1.41	7.8	120	64	44.0	.154	103	6.2
A.C.	F	27	5′2″	127	1.57	3.5	85	47	39.0	.145	70	2.7
A.C.	F	27	5′2″	127	1.57	3.5	109	69	28.0	.126	84	3.6
J.C.	M	35	5′7″	133	1.70	6.0	148	88	35.0	.143	90	4.2
A.G.	F	30	5′4″	108	1.49	3.6	83	33	33.0	.142	72	2.2
H.M.	M	34	5′4″	172	1.82	3.5	120	80	43.0	.153	63	3.0
M.S.	F	30	5′1″	111	1.47	2.8	134	75	29.0	.132	60	3.0
C.L.	F	26	5′10″	258	2.23	2.3	115*	70*	33.0	.140	79	2.3
C.L.	F	26	5′10″	258	2.23	2.4	115*	70*	32.0	.140	70	2.0
B.H.	M	29	5′9″	168	1.91	2.7	120*	72*	49.5	.152	66	3.2

* By cuff.

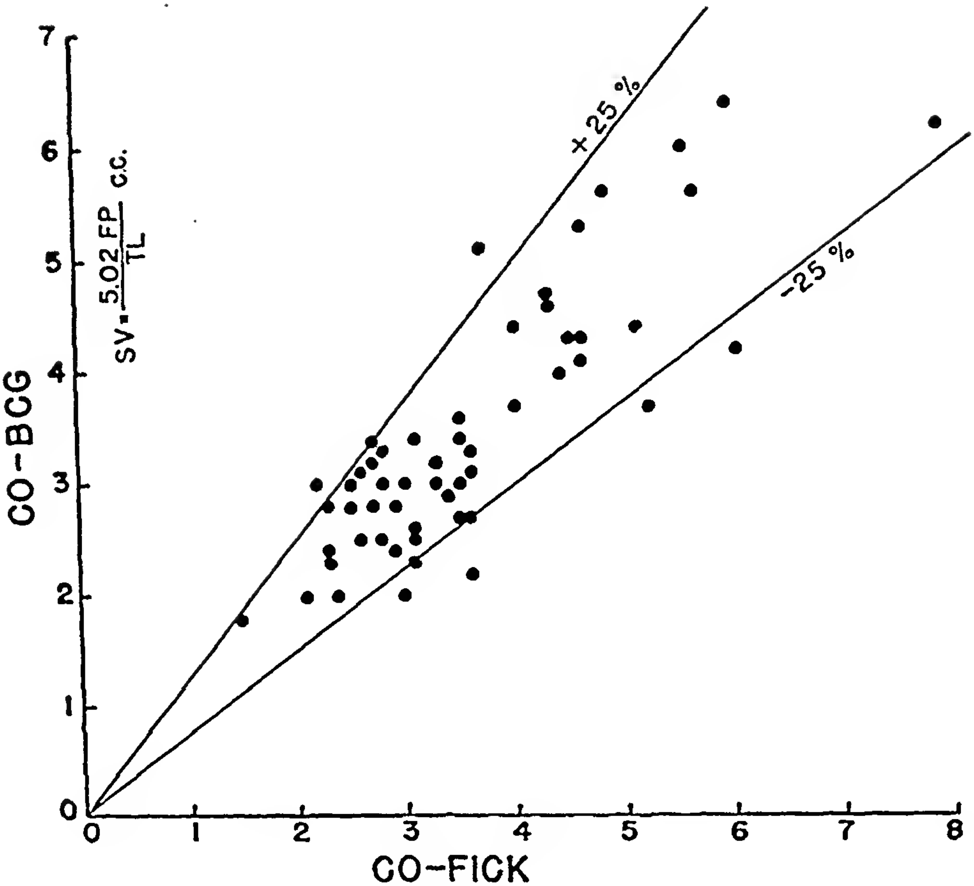

FIG. 3. CARDIAC INDICES COMPUTED FROM THE BALLISTIC TRACING PLOTTED AGAINST THE VALUES DETERMINED FROM THE CATHETER METHOD FOR PATIENTS IN WHOM THERE IS NO CLINICAL EVIDENCE OF CARDIAC DISEASE

The limits of ± 25 per cent deviation from the catheter values are shown.

catheter technique and is subject, therefore, to any fundamental errors in that method.

Fifty-four of the observations reported here were made on 32 subjects without clinical evidence of heart disease. The data on this group are given in Table II. These subjects represent a cross-section of the hospital population. They include members of the staff, convalescent patients, patients with anemia, thyrotoxicosis and shock, and normal subjects given albumin and saline intravenously. If the subjects were cooperative, and if the technical procedures were satisfactory, the data were used, regardless of whether or not the subject showed evidence of anxiety or apprehension. We are primarily interested in determining the relation of the ballistic tracings to the cardiac output as measured by the catheter technique under a variety of conditions, rather than in establishing basal values for the cardiac output. The comparison of the cardiac indices as measured by the 2 methods is shown for this group of subjects in Figure 3. Eighty-seven per cent of the values obtained by the ballistic method fall within 25 per cent of those by the catheter method. The correlation coefficient relating the data from the 2 methods has a value of 0.83.

Twenty-seven almost simultaneous measurements were made on a group of 26 patients with heart disease or myxedema. The data on these patients, with diagnosis, are given in Table III. The cardiac output as measured by the 2 methods did not agree as closely in this group as in the group without heart disease. Excepting those patients having aortic insufficiency, 60 per cent of the ballistic values of the patients in this group fell within 25 per cent of the catheter values (Figure

4). As one might expect on theoretical grounds, the patients with aortic insufficiency failed to show good correlation between the 2 methods. While the ballistic outputs were large, the catheter outputs were small, the difference between the ballistic and the catheter results giving some indication of the quantity of blood regurgitated during each cardiac cycle.

With the exception of those patients with aortic insufficiency, the cardiac output as calculated from the ballistic record fell within 25 per cent of that obtained by the catheter method in 80 per cent of the cases for the entire group studied.

Serial measurements were made at various stages in the treatment of 5 patients in shock (Figure 5). In Figure 6 portions of 4 successive ballistic records taken on one such patient are shown. All patients had distinct clinical evidence of shock. Two (W.T. and M.Z.) were patients with hemorrhagic shock from superficial stab wounds, one (L.A.) with a penetrating stab wound of the chest, one (E.J.) following an operative procedure for tuberculous infection of the kidney, and one (S.M.) with shock resulting from hemorrhage after drainage of a periurethral abscess. All, except one whose arterial pressure was 103/59, had systolic blood pressures below 100 mm. Hg at the time the initial measurements were made. The increase in cardiac output after treatment with saline and albumin was shown by both methods of measurement, and the agreement between the methods was good. In fact it appears that changes

TABLE III

Subject	Sex	Age	Ht.	Wt.	S.A.	Fick CI	Pressure		F.	T.	HR	BCG CI	Diagnosis
							P_s	P_d					
		yrs.		*lb.*	*sq. m.*	*l. per min. per sq. m.*	*mm. Hg*		*grams*	*sec.*	*per min.*	*l. per min. per sq. m.*	
P.B.	F	25	4′10″	108	1.30	3.0	155	82	16.0	.148	64	2.0	Treated myxedema, dilated heart
L.W.	F	42	5′4″	137	1.66	1.9	100*	70*	24.0	.140	65	1.9	Myxedema
A.D.	F	70	5′7″	108	1.55	5.2	175	72	28.0	.133	124	5.5	Thyrotoxicosis—auricular fibrillation
M.W.	F	45	5′4″	105	1.48	4.3	176	83	13.2	.129	86	2.1	Cardiac enlargement—? cause
A.F.	F	15	5′2″	90	1.35	6.0	123	57	39.0	.138	62	3.9	Sickle cell anemia with cardiac enlargement
M.H.	F	39	5′9″	168	1.91	2.7	129	60	40.0	.149	63	2.5	Rheumatoid arthritis—cardiac enlargement—? cause
I.H.	F	33	5′7″	137	1.71	3.9	116*	66*	41.0	.116	106	6.2	Rheumatic heart disease with mitral stenosis—5 mo. pregnant
C.H.	M	29	5′11″	168	1.94	2.7	152	87	42.0	.136	57	2.8	Cardiac enlargement—? cause
M.W.	F	57	5′5″	150	1.75	1.7	139	87	18.5	.198	88	1.5	Rheumatic heart disease with mitral stenosis
J.S.	M	34	5′10″	138	1.76	3.1	106	45	61.0	.179	58	2.7	Rheumatic heart disease with mitral stenosis
T.E.	M	53	5′7″	137	1.71	1.9	160*	65*	53.0	.255	63	2.4	Coarctation of the aorta
D.M.	M	62	6′	119	1.70	1.8	125	76	36.0	.166	65	2.3	Cor pulmonale—emphysema
J.M.	M	38	5′2″	123	1.54	3.5	105*	70*	30.0	.140	73	3.0	Cor pulmonale
T.S.	M	54	5′8″	133	1.71	2.9	133	72	33.0	.148	60	2.3	Rheumatic heart disease with mitral stenosis—auricular fibrillation
J.M.	F	42	5′2″	105	1.45	2.7	140	106	12.5	.132	182	4.2	Paroxysmal auricular tachycardia
J.W.	M	30	5′3″	115	1.52	2.6	127	60	39.0	.146	54	2.9	Rheumatic heart disease with mitral insufficiency
M.D.	M	14	5′4″	104	1.48	6.3	113	54	55.6	.154	77	5.3	Arteriovenous fistula
J.C.	M	32	6′1″	176	2.00	1.9	135	60	128.0	.168	88	9.0	Aortic insufficiency with failure
J.C.	M	32	6′1″	176	2.00	1.7	131	56	96.0	.191	78	5.2	Aortic insufficiency with failure
J.E.	M	34	5′11″	162	1.91	2.9	165	58	55.4	.162	56	3.0	Aortic insufficiency
W.F.	M	48	5′11″	144	1.82	1.9	168	78	54.0	.215	92	3.9	Aortic insufficiency with failure
L.F.	F	36	5′4″	108	1.50	2.3	182	35	53.7	.270	75	3.2	Aortic insufficiency with failure
G.H.	M	57	5′8″	119	1.64	4.0	148	44	48.4	.156	92	4.9	Aortic insufficiency
A.H.	F	62	5′4″	117	1.55	2.4	174	64	20.0	.160	88	2.4	Aortic insufficiency—well compensated
D.T.	F	27	5′7″	130	1.68	2.0	210	63	66.4	.149	76	7.0	Aortic insufficiency
A.W.	M	52	6′	153	1.89	3.0	175	43	123.0	.160	73	8.5	Aortic insufficiency with failure
H.Y.	M	72	5′7″	159	1.83	2.8	181	56	37.7	.166	80	3.2	Aortic insufficiency

* By cuff.

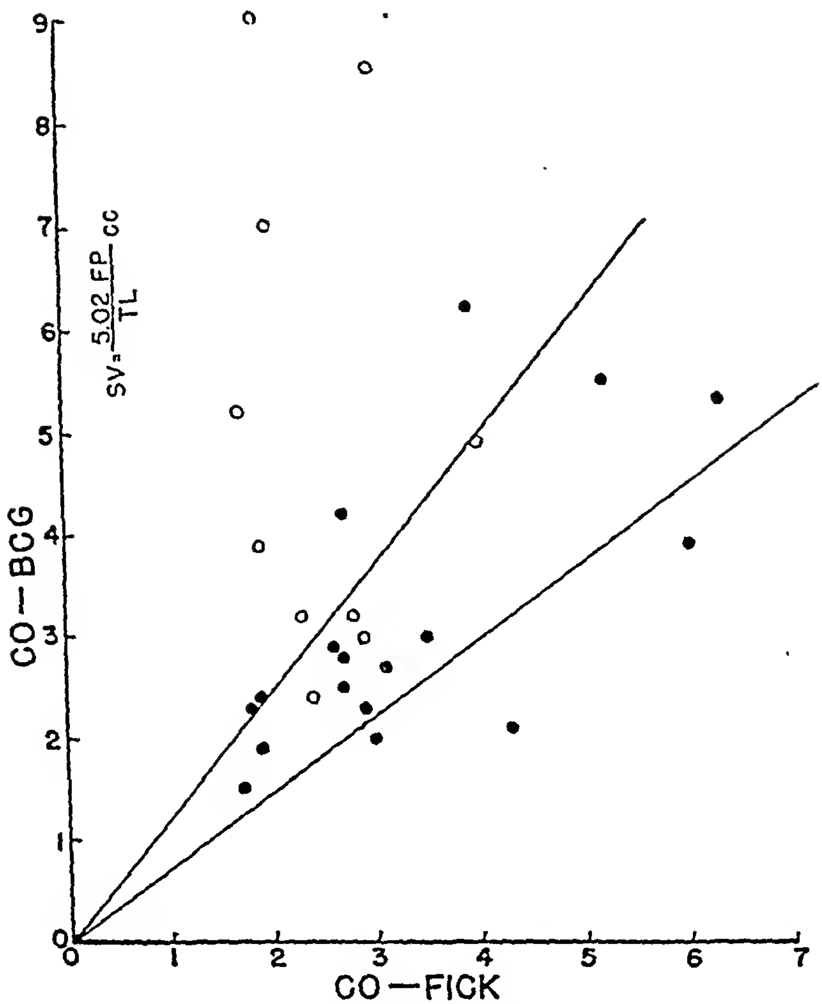

FIG. 4. THE CARDIAC INDICES FROM THE BALLISTIC TRACINGS PLOTTED AGAINST THE CATHETER VALUES FOR ALL SUBJECTS DIAGNOSED AS HAVING CARDIAC DISEASE OR MYXEDEMA

Those patients having aortic insufficiency are specifically designated by open circles. The limits of ± 25 per cent deviation from the catheter values are shown.

in cardiac output for any individual subject were measured with considerable accuracy by the ballistocardiograph.

Variations in arterial pressure, heart rate and age appear to cause no significant variation in the relationship between the cardiac index as measured by the ballistic method and as determined by the catheter method. This is demonstrated in Figure 7 where the ratios of the ballistocardiograph values to the catheter values are plotted against blood pressure, heart rate, and age. This figure includes the patients without heart disease (solid dots) and the patients with heart disease (open dots), except those patients having aortic insufficiency.

DISCUSSION

In comparing the ballistocardiographic determinations of the cardiac output with the method which involves right atrial catheterization and the application of the Fick principle, it must be remembered that the latter method is a procedure subject to considerable error. Although it does not afford an ideal standard for comparative studies, it does, however, appear to offer the best means available. Its limitations have been evaluated in other

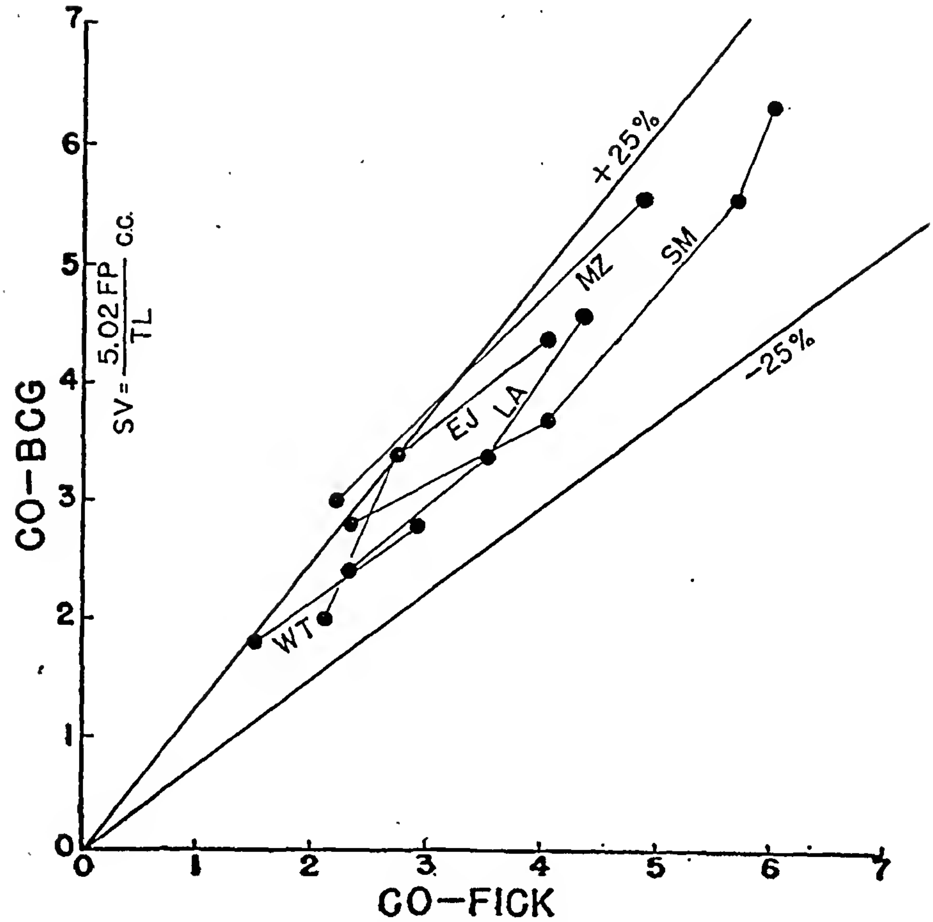

FIG. 5. SERIAL CARDIAC INDEX OBSERVATIONS ON 5 PATIENTS IN SHOCK AND WITH TREATMENT

The limits of ± 25 per cent deviation from the catheter values are shown.

communications from this laboratory (4, 5). Such factors as technical variations in the chemical determinations, inadequate sampling of mixed blood due to the streaming of the blood within the heart, and minute to minute variations of the cardiac output account for unavoidable errors, which may at times rise as high as ± 25 per cent. Moreover, since the computation of the cardiac output depends on the arteriovenous difference, the effect of errors will be larger when this difference is small. These factors should be considered in evaluating this comparative study; and because of these factors even a perfect ballistocardiographic method would fail to give excellent correlation.

The only other reported study comparing the ballistocardiographic method to the catheter technique of cardiac output determination is that of Cournand *et al.* (6). These authors used a high frequency, undamped ballistocardiograph, and found good correlation in relatively normal subjects. We believe that since our patients represent a wider variety of disease conditions, a wider variance of the data is to be expected. In a later publication (7) these authors reported that they were led to discard the high frequency ballistocardiograph for the patients in, or recovering from, shock. This objection to the use of the ballistocardiograph in the study of shock was not found to be applicable to the instrument described in the present paper.

The low frequency, critically-damped ballistocardiograph offers a remarkably simple and rapid method of determining the output of the heart. Rapid changes in output may be followed with ease, thus permitting studies to be made which are not possible by any other method. It is of particular value in following the output changes in an individual subject such as the studies of patients in shock recorded here. The ballistocardiograph, however, is not without disadvantages. As was anticipated, our data demonstrate that the

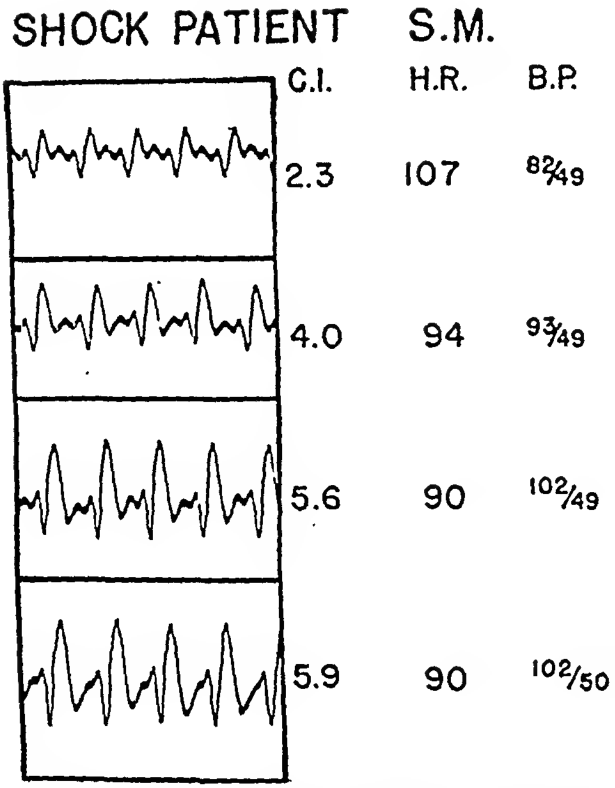

Fig. 6. Portions of the Successive Ballistic Tracings and Some of the Measurements on the Shock Patient S. M. of Figure 5

findings in aortic insufficiency are unrelated to the effective output of the heart. Good results are difficult or impossible to obtain in patients with marked respiratory distress, or those unable to hold their breath for even a few seconds. Extremely irregular heart rhythms and marked tremors might also be mentioned as factors leading to results that are unsatisfactory. Finally, in some patients, particularly those with myocardial disease, we have seen patterns of unusual shape that are quite difficult to interpret.

CONCLUSIONS

The details of the construction of a low frequency, critically-damped ballistocardiograph are presented in this paper, and the procedures for its adjustment to definite physical conditions described. The adjustments enable the reproduction of this type of ballistocardiograph to be made easily, and permit, without the necessity of further calibration by the catheter method, the use of the stroke volume equation tested in this study.

Eighty-one determinations of the cardiac output by the method of right heart catheterization and by almost simultaneous ballistic records with the low frequency, critically-damped ballistocardiograph have been made on a group of 58 normal subjects and hospital patients under a variety of conditions. From the first 50 of these observations on subjects without heart disease, constants were derived for use in determining the cardiac output from the ballistic record. Applying these constants to the entire group of subjects (except patients with aortic insufficiency) the cardiac output computed by the ballistic method fell within 25 per cent of that obtained by the catheter method in 80 per cent of the cases.

Fifty-four of these determinations were made on subjects without evidence of heart disease, but with a variety of other illnesses. Eighty-seven per cent of the results by the ballistic method lay within 25 per cent of the values by the catheter technique. The other twenty-seven patients studied had clinical evidence of heart disease or myxedema. For 17 patients in this group, *i.e.*, excepting those with aortic insufficiency, the cardiac output by the ballistic method was within 25 per cent of that by the catheter technique in 60 per cent of the cases. Patients with aortic insufficiency showed marked discrepancies between the results from the ballistocardiograph and from the catheter method. As might be expected, the ballistic method gave output results which were considerably higher than those by the other method.

Serial measurements on patients in shock and during recovery showed a good correlation between the cardiac output measurements by the 2 methods. These results suggest that changes in cardiac output in any individual subject may be measured with considerable accuracy by the ballistocardiograph.

It is concluded that the low frequency, critically-damped ballistocardiograph provides a useful and reasonably accurate method of determining cardiac output in normal subjects, and in patients with a variety of disease conditions.

We wish to thank Mr. Herman Just of the Department of Physiology, Columbia University College of Physicians and Surgeons, for his precise work in the construction of the ballistic bed, especially the clamps for adjusting the spring lengths. Mrs. Jane Bailey, Miss Eloise Cavin, Miss Maurine Giese and Miss Lois Jackson gave valuable technical assistance.

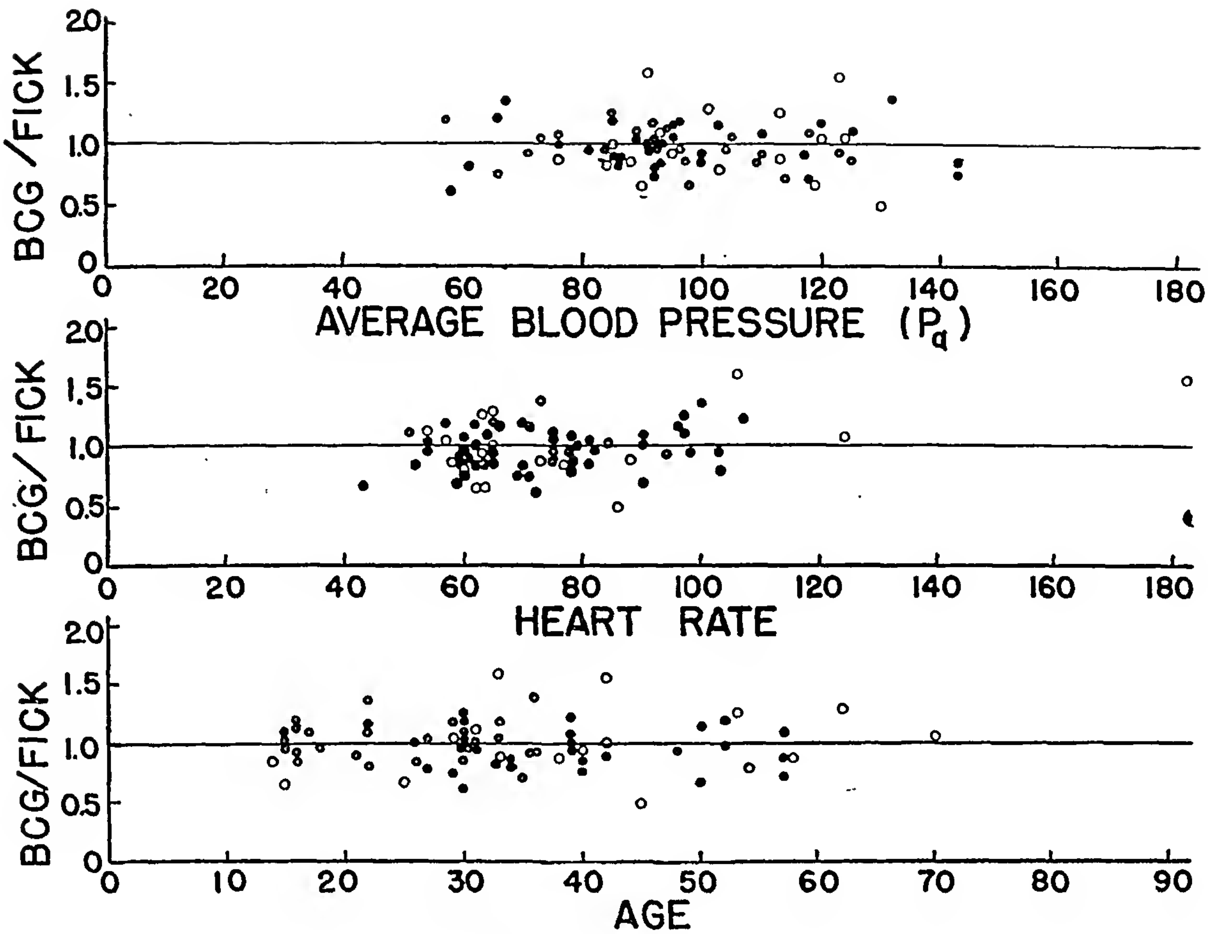

FIG. 7. THE RATIO OF THE BALLISTOCARDIOGRAPH CARDIAC INDEX AND THE FICK CARDIAC INDEX PLOTTED AGAINST THE AVERAGE BLOOD PRESSURE, *Pa*, THE HEART RATE, AND THE AGE OF THE PATIENT

No significant trend with any of these variables is shown.

BIBLIOGRAPHY

1. Nickerson, J. L., and Curtis, H. J., The design of the ballistocardiograph. Am. J. Physiol., 1944, 142, 1.
2. Starr, I., Rawson, A. J., Schroeder, H. A., and Joseph, N. R., Studies on the estimation of the cardiac output in man, and of abnormalities in cardiac function, from the heart's recoil and the blood's impacts; the ballistocardiogram. Am. J. Physiol., 1939, 127, 1.
3. Nickerson, J. L., The low frequency, critically-damped ballistocardiograph. Federation Proc., 1945, 4, 201.
4. Stead, E. A., Jr., Warren, J. V., Merrill, A. J., and Brannon, E. S., The cardiac output in male subjects as measured by the technique of right atrial catheterization. Normal values with observations on the effect of anxiety and tilting. J. Clin. Invest., 1945, 24, 326.
5. Warren, J. V., Stead, E. A., Jr., and Brannon, E. S., The cardiac output in man; A study of some of the errors in the method of right heart catheterization. Am. J. Physiol., 1946, 145, 458.
6. Cournand, A., Ranges, H. A., and Riley, R. L., Comparison of the results of the normal ballistocardiogram and a direct Fick method in measuring cardiac output in man. J. Clin. Invest., 1942, 21, 287.
7. Cournand, A., Riley, R. L., Bradley, W. E., Breed, E. S., Noble, R. P., Lauson, H. D., Gregersen, M. I., and Richards, D. W., Studies on the circulation in clinical shock. Surgery, 1943, 13, 964.

INTUBATION STUDIES OF THE HUMAN SMALL INTESTINE. XXV. THE ABSORPTION OF GALACTOSE FROM THE INTESTINE OF NORMAL INDIVIDUALS AND THYROTOXIC PATIENTS

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The concentration of galactose in the blood stream at determined intervals after its ingestion has been proposed as a clinical test of intestinal absorption (1). It is said not only to indicate the rate at which galactose is absorbed from the intestine, but also to be a means of measuring the ability with which and the rate at which the intestinal mucosa absorbs various substances by the mechanism of phosphorylation (1 to 4). Some workers, finding by this procedure that the thyrotoxic patient shows a higher galactose curve than does the normal subject, have attributed this result to a specific accelerating action on intestinal absorption by the thyroid hormone (2 to 5).

The blood concentration of a substance following its ingestion, however, is not dependent solely on the capacity of the intestinal mucosa to absorb it. First of all, and most important, it is necessary to know whether or not, and to what extent, the material reaches the absorbing surface, and so is available for absorption. The hexoses are absorbed chiefly from the duodenum and jejunum (6 to 9), and from the stomach only when in hypertonic solution (10, 9). Consequently, alterations in gastric and intestinal motility, whether due to organic or functional disturbances, may profoundly affect the availability of the ingested galactose in the absorbing area (6, 29b, 29c). In spite of the experimental evidence that glucose and galactose orally administered are absorbed at a constant rate and independent of their concentration (11, 12, 8), this holds true only within certain limits (13, 6). When widely varying concentrations of a sugar are introduced directly into the intestine the absorption rate is proportional to the concentration (15, 29d, 31, 32, 36).

Secondly, it has been demonstrated in animals that irrespective of the actual rate of hexose absorption, the concentration in the blood is chiefly dependent on metabolic factors (11, 12). In this respect the liver is the principal, though not the sole, organ involved (14, 15, 19b, 24, 35). Hepatic dysfunction especially has been held responsible by some authors for the hypergalactemia found after the ingestion of that sugar in the thyrotoxic patient (29a). Furthermore, in such a condition, as well as in cases with various nutritional, endocrine, and urinary tract disturbances, the renal output of the sugar is often altered (16 to 24, 14, 15). Thus the rate of its excretion constitutes a third factor that influences the concentration of galactose in the blood (24, 29e).

Finally, it has been claimed, as we shall show, that the blood concentration of galactose is in some way affected by the coincident concentration of glucose (15, 16, 20, 21, 25).

In an attempt to obviate some of these disturbing factors, we have employed the absorption technique devised by Nicholson and Chornock (26), which makes possible over a fixed period of time the presence of a known quantity of the galactose in a predetermined area of the intestinal tract. Thus it measures accurately the availability of the galactose for absorption and the actual quantity absorbed, and so eliminates the chief factors that previously, for the human subject, had not been controlled. The other factors, metabolic disturbances and excretion through the kidneys, have been controlled insofar as possible by the selection of the subjects for the experiments.

SUBJECTS AND METHODS

For this study, 6 normal persons and 5 patients with thyrotoxicosis were employed. The normals were paid subjects found to be in good health by history, physical examination, and clinical laboratory studies. Radiological examinations of the gastro-intestinal tract and the basal metabolic rates were within normal limits. The hyperthyroid subjects exhibited clear-cut clinical pictures of thyrotoxicosis, and were patients from the medical wards awaiting thyroid surgery.

The details of the technique developed by Nicholson and Chornock need not be repeated. It involves the injection of the test solution into the mid-duodenum through one lumen of the Miller-Abbott tube (27) and the removal of the residue from the jejuno-ileal area, 90 cm. lower in the tract, through the other lumen.

The subjects fasted 12 hours (overnight) and on the morning of the test day, after getting the apparatus into its proper position, which usually required 2 to 3 hours, the absorption study was undertaken. The absorption period varied from 30 to 90 minutes, and the exact procedure was as follows: A 10 per cent solution of galactose was allowed to drip into the mid-duodenum via the first lumen at a rate of 10 ml. per minute. It was introduced by means of a flask with a Murphy drip attachment at a hydrostatic pressure of 75 cm. The rate of drip was regulated by means of a Hoffman clamp. This concentration of galactose and the rate of its administration were selected after preliminary experiments indicated that they gave the most consistent results.

As soon as the galactose began to flow into the duodenum, aspiration of the succus from the jejunum was begun. All of the material aspirated during the experimental period was collected in flask No. 1 and subsequently analyzed quantitatively for its galactose content. By subtracting this unabsorbed quantity, plus the quantities obtained in flasks 2, 3, 4 and 5 (the details of collection of which are outlined below), from the amount administered, a calculation of the amount absorbed was derived.

After administering the desired quantity of galactose, the absorption period was abruptly terminated by discontinuing the galactose drip, removing flask No. 1 and injecting 30 ml. of a 33 per cent solution of magnesium sulfate into the duodenum. In each experiment, in order to check on the abruptness with which the absorbing period was ended, a 30-minute period of washing the bowel with tap water was carried out. Aspiration was continued using flasks Nos. 2, 3 and 4 for the collection at 10-, 20- and 30-minute periods. Each of the specimens obtained during this wash period was analyzed for galactose. The tap water wash was performed in the same manner as the galactose was administered.

As can be seen by reference to Table I, except in specimen No. 2, which immediately followed the magnesium sulfate injection, only traces of galactose were present after the experimental period, thus indicating that the peristaltic rush induced by the magnesium sulfate solution was highly effective in abruptly terminating the absorption period. The intensity of this peristaltic rush, and the completeness with which it cleared the intestinal lumen, were confirmed in a few experiments by adding vital red in known concentration to the magnesium sulfate solution and determining its time of appearance; this was from 1 to 3 minutes. Furthermore, it was demonstrated that most of the dye and the sugar contained in specimen

TABLE I

The absorption of galactose from a 90-cm. segment of the human duodeno-jejunum during its administration at a fixed rate into the duodenum in normal and thyrotoxic individuals

Subject	Galactose administered in 10 per cent solution	Experimental period	Galactose recovery					Galactose absorbed	Remarks
			Absorption period.	Following $MgSO_4$ injection					
			Spec. 1	Spec. 2	Spec. 3	Spec. 4	Spec. 5		
	grams	*min.*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	
H.M.	30.0	30	9.20	0.98	0.11	0.02	0.00	19.7	Control subject
G.C.	30.0	30	1.60	2.82	0.00	0.00	0.00	25.6	Control subject
M.B.	30.0	30	6.89	0.82	0.01	0.00	0.00	22.3	Control subject
C.B.	30.0	30	8.53	3.64	0.09	0.00	0.02	17.7	Control subject
D.N.	30.0	30	10.23	4.10	0.09	0.00	0.00	15.6	Control subject
G.C.	60.0	60	22.40	2.00	0.20	0.02	0.00	35.4	Control subject
M.B.	60.5	60	9.61	0.72	0.21	0.00	0.06	50.0	Control subject
H.M.	55.5	60	22.80	2.41	0.05	0.01	0.00	30.2	Control subject
C.B.	91.0	90	29.47	3.28	1.09	1.02	0.00	56.2	Control subject
W.J.A.	30.0	30	11.18	0.79	0.00	0.00	0.10	17.9	Thyrotoxic patient
H.A.	30.0	30	5.60	4.90	0.00	0.00	0.00	19.5	Thyrotoxic patient
M.T.	30.0	30	8.28	6.13	1.53	0.62	0.00	13.4	Thyrotoxic patient
D.W.	30.0	30	2.82	6.53	0.86	0.58	0.01	19.2	Thyrotoxic patient

TABLE II

The absorption of galactose from the duodeno-jejunum per square meter of body surface per ½ hour in normal and thyrotoxic individuals

Subject						Galactose			
Initials	Sex/Age	Height	Weight	Body surface area	Basal metabolic rate	Administered by tube	Experimental period	Total absorbed	Amount absorbed per sq. meter per 30 min.
		inches	*lbs.*	*sq. meters*	*per cent*	*grams*	*min.*	*grams*	*grams*
H.M.	M/29	65	148	1.74	−18	30 60 55.5	30 60 60	19.7 35.8 30.2	11.4 10.2 8.8
G.C.	M/31	72	201	2.12	−8	30 30 60	30 30 60	25.6 25.5 35.4	12.0 12.1 8.8
M.B.	F/38	61½	133	1.59	−3	30 60.5 59.7 30 30	30 60 60 30 30	22.3 50.0 41.9 24.7 25.0	14.2 15.6 13.0 15.5 15.7
C.B.	F/60	61½	102½	1.42	+3	30 30 52.5 60 91	30 30 60 60 90	17.7 18.7 29.1 25.5 56.2	12.2 13.2 10.0 9.0 13.2
D.N.	F/23	66	126	1.62		30	30	15.6	9.6
S.B.	F/30	66	110	1.53		61	60	44.4	14.5
H.A.*	F/56	60	125½	1.52	+42	30	30	19.5	12.8
W.J.A.*	M/51	65¾	110	1.53	+30	30	30	17.9	11.8
M.T.*	F/49	67	106	1.53	+30	30	30	13.4	8.7
D.W.*	F/47	61½	108	1.45	+18	30	30	19.2	13.2
L.M.*	F/30	72	137½	1.81	+38	30	30	26.1	14.4

* Thyrotoxic patient.

No. 2 appeared within the first 5 minutes of the 10-minute period of aspiration.

As a further check in every experiment, a fifth specimen (No. 5) was collected and analyzed. This consisted of the material that could be aspirated from the duodenum and stomach during the time the tube was being withdrawn. When any considerable amount of galactose was detected in this specimen, the experiment was discarded.

The absorption studies were conducted in the beginning over periods of 30, 60 and 90 minutes. As these revealed the rate of absorption to be fairly constant, a 30-minute period was chosen as satisfactory for the experiments comparing the results in normal and thyrotoxic patients (Tables I and II).

In addition to comparing the rate of galactose absorption in normal and hyperthyroid patients by this method, blood specimens were drawn at ½-, 1- and 2-hour periods after beginning the galactose administration, to determine the concentration of galactose in the blood stream (Tables III and IV). Thus a comparison of the blood galactose values of normal and of hyperthyroid individuals was accomplished simultaneously with the measurement of the amount of galactose absorbed from the intestine.

In a small group of experiments, a solution of glucose was administered ½ hour prior to the administration of the galactose solution, using the same technique of administration as was employed for the galactose. This modification of the experiment was employed in order to determine what effect this previous administration of glucose would have on the absorption of the galactose from the intestine, and on the concentration of galactose in the blood (Table V).

In every instance the chemical determinations on the blood and succus entericus specimens were conducted promptly after their collection.

Determination of blood galactose.

The blood specimens were collected in thymolfluoride tubes. Haden's methods for protein precipitation and for

TABLE III

A comparison of the blood galactose concentration with the amount of galactose absorbed during 30- and 60-minute experimental periods in normal and thyrotoxic individuals

Subject	Galactose administered	Experimental period	Galactose absorbed	Galactose absorbed per sq. meter per ½ hr.	Blood galactose concentration		
					½ hour	1 hour	2 hours
	grams	*min.*	*grams*	*grams*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
G.C.	30	30	25.5	12.1	88	69	
M.B.	59.7	60	41.9	13.0	52	67	
	60	60	50.0	15.6	52	66	9
	30	30	24.6	15.5	58	22	
H.M.	60	60	35.8	10.2		47	11
C.B.	60	60	25.5	9.0	37		
	52	60	29.1	10.0		72	40
	30	30	18.7	13.2	42	29	
	22.5*	30	21.5	15.0	42	15	
	45.0†	30	29.	20.0	55	42	
H.A.‡	30	30	19.6	12.8	60	17	
M.T.‡	30	30	13.4	8.7	10	18	
W.J.A.‡	30	30	17.9	11.8	100	23	
D.W.‡	30	30	19.2	13.2	16	17	
L.M.‡	30	30	26.1	14.4	58	22	

* 7.5 per cent solution.
† 15 per cent solution.
‡ Thyrotoxic patient.

the preparation of whole blood filtrates were employed. The filtrates were then freed of glucose by means of yeast fermentation, by the method of Somogyi. Control tubes of glucose and of yeast suspension were simultaneously incubated with the blood filtrates. After adequate fermentation, the filtrates were centrifuged and filtered. The concentration of galactose was then determined in the filtrate by the method of Benedict, using a Klett-Summerson Photoelectric Colorimeter.

Determination of galactose in succus entericus.

The volume of each sample was measured. To 2 ml. of each sample were added 17 ml. of H_2O, 0.5 ml. of 10 per cent $ZnSO_4$, and 0.5 ml. of 0.5 N NaOH. This was shaken and filtered. The excess zinc was removed by adding a pinch of anhydrous Na_2CO_3, shaking well and filtering. In most instances the first 2 samples were diluted further, so that the final dilution was 1 to 100. Two ml. of each of the diluted samples were then analyzed for galactose.

RESULTS AND DISCUSSION

This method of studying the absorption of sugar from the duodeno-jejunal area was highly satisfactory in that it allowed control of the amount, concentration and time during which the sugar was delivered to a selected area of the intestine. The absorbing or experimental period, furthermore, could be abruptly terminated, an accomplishment not satisfactorily attained by other methods employable in the human subject (Table I).

Galactose, when given in 10 per cent solution, was found to be absorbed at a fairly constant rate in the same subject in repeated tests extending over intervals of 30, 60 and 90 minutes (Tables I and II) and varied between 13.4 and 25.6 grams per ½ hour for the different subjects. Calculating the grams absorbed on the basis of body surface area per ½ hour gave a closer correlation to serve as a basis for comparison in the different individuals (29d). The rates calculated on this basis were found to range from 8.7 to 15.7 grams per square metre of body surface per ½ hour, with an average value of 11.2 grams (Tables II and III).

No differences were observed between the rates at which the galactose was absorbed in 6 healthy and in 5 thyrotoxic patients, nor was there any notable difference in the blood galactose concentration of the 2 groups (Tables III and IV).

Furthermore, as can be seen by reference to Table III, there was no direct correlation between the blood galactose concentration and the amount of that sugar absorbed during a ½ hour period of its administration. When the administration was continued for a full hour, the second or 1-hour blood specimen, showed a somewhat higher galactose concentration than when the galactose was instilled for only ½ hour. The blood galactose concentrations obtained during the longer period of administration tended to resemble those after the oral administration of the sugar (Tables IV and V). This provides experimental proof that a variation in gastric motility, by increasing or decreasing the amount of galactose available for absorption, materially alters its concentration in the blood, even though the ability to absorb the sugar from the duodeno-jejunal area remains constant.

In view of these observations, serious doubt necessarily arises as to what, if any, value the glactose tolerance test has as a means of arriving at an index of intestinal absorption. Certainly, on the basis of these experiments, it seems valueless.

TABLE IV

A comparison of the blood galactose concentration after its oral administration, and after its intra-duodenal administration in normal and thyrotoxic individuals

Subject	Galactose administered	Method of administration	Blood galactose concentration: After ½ hour	After 1 hour	After 2 hours	Remarks
	grams		*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	
G.C.	40	Oral	38	16	10	Control subject. Urinary excretion, 0.68 gram in 5 hrs.
	30	Tube	88	69		
M.B.	40	Oral	62	45	24	Control subject. Urinary excretion, 1.5 grams in 5 hrs.
	60.5	Tube	52	66	9	
	30	Tube	58	22		
C.B.	40	Oral	77	110	84	Control subject. Urinary excretion, 1.07 grams in 5 hrs.
	22.5	Tube	42	15		
	60	Tube	37			Urinary excretion, 0.4 gram in 5 hrs.
	30	Tube	42	29		
	45	Tube	55	42		
L.D.	40	Oral	39	59	8	Control subject
L.M.	30	Tube	58	22		Thyrotoxic patient. Urinary excretion, 0.58 gram in 5 hrs.
W.J.A.	40	Oral	38	48		Thyrotoxic patient
	30	Tube	100	23		
D.W.	40	Oral	20	37		Thyrotoxic patient
	30	Tube	16	17		

When glucose was given ½ hour prior to the administration of galactose, no matter whether the 2 sugars were administered orally or intra-duodenally, a considerable lowering of the concentration of the galactose in the blood occurred (Table V). This effect was not due to any decrease in the amount of galactose absorbed, thus indicating that the lowered blood concentration resulted from some other influence exerted by the glucose. These observations confirm those previously made, chiefly on experimental animals, which indicate that the metabolism of galactose depends in some way on the availability of glucose (20, 25).

TABLE V

The effects of the previous administration of glucose on the blood galactose concentration in normal subjects

Subject	Galactose administered	Method of administration	Galactose absorbed per sq. meter of body surface area per ½ hour	Blood galactose concentration: ½ hour	1 hour	2 hours
	grams		*grams*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
G.C.	40	Oral		38	16	10
	30	Tube	12.1	88	69	
	30*	Tube	12.0		12	
M.B.	40	Oral		62	45	24
	30	Tube	15.5	58	22	
	30*	Tube	14.2		17	
C.B.	40	Oral		77	110	84
	30*	Oral		2	17	13
	30	Tube	13.2	42	29	
	30*	Tube	12.2		16	

* In this instance 30 grams of glucose were administered ½ hour before the galactose and by the same route.

The values obtained by us for the concentration of galactose in the blood of normal subjects were higher than those reported by Althausen (5), but they were of the same order of magnitude as have been reported by other observers (28, 29a, 30).

CONCLUSIONS

1. Employing the technique of Nicholson and Chornock in a study of the absorption of a 10 per cent solution of galactose from the upper human intestine, administered at a rate of 10 ml. per minute, the rate was found to range from 8.8 to 15.7 grams per square meter of body surface area per ½ hour, with an average of 11.2 grams.

2. The thyrotoxic patient, under identical conditions, does not absorb galactose any faster than the healthy individual.

3. The degree of galactemia bears no constant relation to the amount of galactose absorbed from the intestine in either the normal subject or the thyrotoxic patient.

4. Galactemia may be depressed by the previous administration of glucose without any decrease in the absorption of the galactose. This tends to provide further evidence to Folin's, Berglund's (20) and Bodansky's (25) statements that the retention and utilization of galactose by the body depend to a large extent on the availability of glucose and are not due to any decrease in absorption of the galactose as was thought to be the explanation in animal experiments (11, 12).

BIBLIOGRAPHY

1. Althausen, T. L., A test for intestinal absorption. Am. J. Digest. Dis. & Nutrition, 1939, 6, 544.
2. Althausen, T. L., The disturbance of carbohydrate metabolism in hyperthyroidism. J. A. M. A., 1940, 115, 101.
3. Althausen, T. L., and Stockholm, M., Influence of the thyroid gland on absorption in the digestive tract. Am. J. Physiol., 1938, 123, 577.
4. Althausen, T. L., and Wever, G. K., Galactose tolerance in hyperthyroidism. J. Clin. Invest., 1937, 16, 257.
5. Althausen, T. L., Lockhart, J. C., and Soley, M. H., A new diagnostic test (galactose) for thyroid disease. Am. J. M. Sc., 1940, 199, 342.
6. Abbott, W. O., Karr, W. G., Glenn, P. M., and Warren, R., The absorption of glucose from the duodenum. Am. J. M. Sc., 1940, 200, 532.
7. Karr, W. G., Abbott, W. O., Hoffman, O. D., and Miller, T. G., The concentration and movement of glucose solutions in the stomach and duodenum. Am. J. M. Sc., 1940, 200, 524.
8. Verzar, F., and McDougall, E. J., Absorption from the Intestine. Longmans, Green and Co. Ltd., London, England, 1936.
9. Warren, R., Karr, W. G., Hoffman, O., and Abbott, W. O., The absorption and expulsion of glucose from the stomach. Am. J. M. Sc., 1940, 200, 639.
10. Morrison, J. L., Shay, H., Ravdin, I. S., and Cahoon, R., Absorption of glucose from the stomach of the dog. Proc. Soc. Exper. Biol. and Med., 1939, 41, 131.
11. Cori, C. F., The influence of insulin on the utilization of glucose, fructose, and dihydroxyacetone. J. Biol. Chem., 1928, 76, 755.
12. Cori, C. F., The fate of sugar in the animal body. J. Biol. Chem., 1925, 66, 691.
13. Groen, J., The absorption of hexoses from the upper part of the small intestine in man. J. Clin. Invest., 1937, 16, 245.
14. Bollman, J. L., Mann, F. C., and Power, M. H., The utilization of galactose following complete removal of the liver. Am. J. Physiol., 1935, 111, 483.
15. Duel, H. J., Jr., Intermediary metabolism of fructose and galactose. Physiol. Rev., 1936, 16, 173.
16. Banks, B., Sprague, P., and Snell, A., Clinical evaluation of the galactose tolerance test. J. A. M. A., 1933, 100, 1987.
17. Bassett, A. M., Althausen, T. L., and Coltrin, G., A new intravenous galactose clearance test for differentiation of obstructive from parenchymatous jaundice. Proc. Soc. Exper. Biol. and Med., 1940, 45, 405.
18. Bloch, J., and Weisz, M., Reflex-oder Resorptionshyperglykamie. Ztschr. f. d. ges. Exp. Med., 1930, 69, 453.
19a. Budak, M., Testing of liver function by means of intravenous galactose solution. Chem. Abstracts, 1933, 27, 3732.
19b. Cori, C. F., and Cori, G. T., The carbohydrate metabolism of adrenalectomized mice and rats. J. Biol. Chem., 1927, 74, 473.
20. Folin, O., and Berglund, H., Some new observations and interpretations with reference to transportation, retention and excretion of carbohydrates. J. Biol. Chem., 1922, 51, 213.
21. Harding, V. J., and Van Nostrand, F. H., Variations in blood and urinary sugar after ingestion of galactose. J. Biol. Chem., 1930, 85, 765.
22. Jankelson, I. R., and Lerner, H. H., Intravenous galactose liver function test. Am. J. Digest. Dis. and Nutrition, 1934, 1, 310.
23. Pollack, S., Beitrag zur Leberfunktionsprufung mittels intravenoser Kalaktoselosung. Wien. Arch. F. inn. Med., 1932, 22, 317.
24. Tumen, H. J., and Piersol, G. M., The value of alimentary galactosuria in the diagnosis of jaundice. Ann. Int. Med., 1933, 7, 311.
25. Bodansky, M., Fructose, glucose and galactose tolerance in dogs. J. Biol. Chem., 1923, 56, 387.
26. Nicholson, J. T. L., and Chornock, F. W., Intubation studies of the human small intestine. XXII. An improved technique for the study of absorption; its application to ascorbic acid. J. Clin. Invest., 1942, 21, 505.
27. Miller, T. G., and Abbott, W. O., Intestinal intubation: a practical technique. Am. J. M. Sc., 1934, 187, 595.
28. Harding, V. J., and Grant, G. A., Metabolism of galactose. J. Biol. Chem., 1933, 99, 629.
29a. MacLaglan, N. F., and Rundle, F. F., Liver function in thyrotoxicosis. Quart. J. Med., 1940, 215, July.
29b. May, C. D., and McCreary, J. F., The glucose tolerance test in celiac disease. J. Ped., 1940, 17, 1943.

29c. Marrazzi, R., The influence of adrenalectomy and of fasting on the intestinal absorption of carbohydrates. Am. J. Physiol., 1940, 131, 36.

29d. McKay, E. M., and Bergman, H. C., The rate of absorption of glucose from the intestinal tract. J. Biol. Chem., 1933, 101, 453.

29e. Meczner, L., A combined liver and kidney functional test by means of the galactose test. Chem. Abstracts, 1933, 27, 3733.

30. Roe, J. H., and Schwartzman, A. S., Galactose tolerance in normal and diabetic subjects and the effect of insulin upon galactose metabolism. J. Biol. Chem., 1932, 96, 717.

31. Pierce, H. B., The absorption and utilization of carbohydrates. J. Nutrition, 1935, 10, 689.

32. Ravdin, I. S., Johnston, C. G., and Morrison, P. J., Comparison of concentrations of glucose in stomach and intestine after intragastric administration. Proc. Soc. Exper. Biol. and Med., 1933, 30, 955.

35. Rowe, A. W., The metabolism of galactose; III. The influence of disturbed endocrine function. Am. J. M. Sc., 1935, 190, 686.

36. Shay, H., Gershon-Cohen, J., Fels, S. S., and Monroe, F. L., The absorption and dilution of glucose solutions in the human stomach and duodenum. Am. J. Digest. Dis. & Nutrition, 1939, 6, 535.

THE NUTRITIONAL REQUIREMENTS FOR NITROGEN BALANCE IN SURGICAL PATIENTS DURING THE EARLY POSTOPERATIVE PERIOD[1]

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The role of protein nutrition in the convalescence of surgical patients has been emphasized by many authors during the past 15 years, and much of this work has recently been ably reviewed by Lund (1). The observations of Cuthbertson (2), confirmed by Howard and his co-workers (3), that fractures of long bones resulted in a marked negative nitrogen balance which could not be overcome by substantial increases in the protein intake, raised certain questions regarding the possible effects on nitrogen requirements and nitrogen balance of extensive surgical operations. It seemed important to know how soon after operation a positive nitrogen balance could be re-established, and what levels of nitrogen and caloric intake would be required to accomplish this.

Many of the previous concepts of the protein nutrition of surgical patients have been based on changes which were found in the serum protein concentration at different periods. It is generally acknowledged that the use of such data may lead to erroneous conclusions, but they have continued to be used by many investigators chiefly because of the difficulties in carrying out the more informing balance studies. In this project, additional technical aid made it possible for us to carry out both types of study. Our results indicate that the serum protein concentration may, at times, increase in spite of a negative nitrogen balance during the period of study.

It has also been possible to compare the effectiveness of whole protein with various preparations of hydrolyzed protein in the maintenance of the patient's nutrition after an operation on the gastro-intestinal tract.

PROCEDURE

In selecting patients, we gave preference to young adult males, but we did not limit the study to this group. The patients selected had usually undergone an extensive surgical operation, and usually one which interfered, at least to some extent, with the ability to eat. Thus, preference was given to patients who had had a gastric resection, and to neurosurgical patients who had had a craniotomy or craniectomy. The latter group will be considered in more detail in a separate paper, but the entire series is presented in Table I.

In the gastric cases, Abbott-Rawson tubes were frequently placed at operation either according to the oro-jejunal method of Stengel and Ravdin (4) or according to the gastro-jejunal method of Bisgard (5). In the craniotomy cases, Levine tubes were often passed into the stomach. Some patients in both groups were fed by mouth.

The various types of feeding used may be listed as follows:

1. Food from a metabolic kitchen, either in the form of a liquid, soft or full hospital diet.
2. Amigen (enzymatic digest of casein and pancreas).[2]
3. Amigen and hospital diet.
4. Lactalbumin hydrolysate.[3]
5. Gastrostomy mixture prepared in the following proportions:

Skim milk	500 ml.
Skim milk powder	50 grams
Cottage cheese	50 grams
Soybean flour	50 grams
Egg	1

Preparation of feedings.

The desired amount of Amigen was dissolved in water and either glucose, starch or Dextrimaltose added to make

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Pennsylvania.

[2] Amigen powder (Mead Johnson & Co.).

[3] Wyeth and Co.

up the predetermined number of calories. In some patients the Amigen was given in water, and additional calories were furnished by a glucose solution given intravenously. The volume of solution given by mouth or tube was approximately 200 ml. at a single feeding, and the frequency of feeding was varied so as to provide the total amount of nitrogen and the calories desired in any 24-hour period. Usually 200 ml. were given every 2 to 3 hours. Lactalbumin hydrolysate was administered in the same manner as Amigen. The gastrostomy mixture was prepared in the laboratory, and carbohydrate was added to provide the desired number of calories.

In the early part of the work the patients were studied for 10 days postoperatively. It became apparent that the usual negative nitrogen balance found following operation became a positive balance as the patients began to eat larger quantities of food. In the latter part of our work, therefore, we limited our nitrogen balance studies to the first 5 days of the postoperative period.

Methods of administration.

When food was given by mouth, it was offered at regular hours, and records were kept of the food actually ingested. When the patient was on a constant intake, the portions not eaten at the regular meal hour, or equivalent substitutes, were offered between meals.

When the patient was fed by tube, the feeding was administered by the drip method. Small portions (usually 200 ml.) were given at intervals of 2 to 3 hours throughout the day. When the gastrostomy mixture was too thick to run by gravity through small tubes, the feedings were injected slowly into the tube by syringe.

Collection of specimens.

All urine was collected under toluene for 24-hour periods throughout the period of study, and sent to the laboratory where volume measurements were made.

Feces were collected in many, but not in all cases; but when they were, the patient was given carmine by mouth at the beginning of the study and at the end of 5 days. Feces were collected as 24-hour specimens from the day of appearance of the first carmine up to and including the day of appearance of the second carmine.

Vomitus was collected and analyzed for total nitrogen. Material obtained by suction drainage was carefully collected and analyzed.

Methods of analysis.

All total nitrogen determinations were done by a semimicro Kjeldahl method (6a and b) and nonprotein nitrogen by a colorimetric method using Nessler's reagent (7).

Samples of Amigen, of lactalbumin hydrolysate, and of the gastrostomy mixture were analyzed for nitrogen. Food furnished as "Hospital Diet" was not analyzed in the laboratory, but values as given in standard diet tables were used (8a and b). Urine was usually diluted 1:50 or 1:100, plasma 1:10, and appropriate samples taken for analysis. Feces were thoroughly mixed by mechanical stirring in acidified water, and an appropriate sample taken for analysis.

Hematocrit readings were determined in Sanford-Magath tubes, using heparin as the anticoagulant.

RESULTS

Nitrogen balance.

Table I shows the results of studies on 55 patients fed by mouth or tube. The results are arranged in order of an increasing nitrogen intake. It is evident that:

1. Thirty-seven (67 per cent) of the 55 patients were in negative nitrogen balance for the first 5 postoperative days; 5 were in equilibrium,[4] and 13 were in positive nitrogen balance.

2. Of 18 in equilibrium or in positive nitrogen balance, 12 fell in the group receiving 0.3 gram of nitrogen per kgm., and 30 calories per kgm. or more daily.

3. Of 18 getting 0.3 gram of nitrogen or over, 12 were in positive nitrogen balance; 6 were in negative nitrogen balance. Of these 6, 3 were on low caloric intakes.

Type of operation.

Most of the patients studied fell into two groups, (*1*) gastric and (*2*) cranial operation. Dividing the patients into these 2 groups failed to show any gross difference in the results of feeding on nitrogen balance; *i.e.*, in both groups, most of the patients who were in balance received 0.3 gram of nitrogen and 30 calories per kgm. daily, or over.

Age and sex.

No correlation was found between age or sex of patients and their ability to attain nitrogen equilibrium with comparable nitrogen and caloric intake.

Nitrogen losses in feces.

There was considerable variation between patients in the amount of nitrogen excreted in the feces. Table II gives figures for the range of excretion and average of a number of patients grouped according to the method of feeding. Those patients who were fed by tube into the jejunum showed the greatest average loss of ni-

[4] If the patient had a daily average nitrogen balance of −1.0 to 0.0, he was considered to be in nitrogen equilibrium.

TABLE I

Patient	Operation	Diet	Nitrogen per intake			Nitrogen output 5 day period					Nitrogen balance	
			N per kgm. daily	Cal. per kgm. per day	Total N 5 days	Urine	Feces	Drain-age	Vomi-tus	Total	Total 5 days	Daily aver.
			grams		*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
Co.	Rankin resect.	Glucose+hosp.	0.03	2	10.8	41.1	5.0*	1.3	0	47.5	−36.7	− 7.3
Ca.	Gastric resect.	Glucose+hosp.	0.03	9	9.7	32.3	4.0	3.7	0	40.0	−30.3	− 6.0
Fa.	Gastric resect.	Hospital	0.03	11	8.0	45.6	4.0	4.7	0	54.3	−46.3	− 9.3
Fri.	Expl. lap.	Glucose+hosp.	0.03	14	10.9	55.4	0.7	0.9	0	57.0	−36.1	− 7.2
Fe.	Gastro-enterost.	Hospital	0.04	20	8.5	26.7	4.0	1.8	0	32.5	−24.0	− 4.8
Ha.	Gastric resect.	Glucose+hosp.	0.05	15	12.1	31.4	3.0	8.3	0	42.7	−30.6	− 6.1
Le.	Gastric resect.	Hospital	0.08	13	28.3	69.3	7.5	2.5	0	79.3	−51.0	−10.2
Ri.	Craniotomy	Hospital	0.09	24	33.3	89.3	1.5	0	0	90.8	−57.5	−11.5
Or.	Gastric resect.	Gastrostomy mix.	0.09	9	26.7	64.5	16.0	1.0	0	81.5	−54.8	−10.9
Ro.	Gastric resect.	Hospital	0.09	30	24.5	34.3	5.0*	0	0	39.3	−14.8	− 2.9
Ei.	Gastric resect.	Hospital	0.10	14	36.1	69.8	5.0*	0	0	74.8	−38.7	− 7.7
Cot.	Craniotomy	Hospital	0.10	18	34.7	73.2	5.0*	0	0	78.2	−43.5	− 8.7
Wa.	Gastric resect.	Hospital	0.13	19	42.0	62.3	3.5	1.0	0	66.8	−24.8	− 4.9
Sm.	Craniotomy	Hospital	0.13	21	57.2	68.3	5.0*	0	0	73.3	−16.1	− 3.2
Ho.	Craniotomy	Gastrostomy mix.	0.14	20	41.2	64.9	2.0	0.5	0	68.9	−27.7	− 5.5
Jo.	Craniotomy	Hospital	0.14	22	40.7	69.7	3.5	0	0.1	73.3	−32.6	− 6.5
Th.	Gastric resect.	Hospital	0.14	22	64.0	108.3	5.0*	0.5	0	113.8	−49.8	− 9.9
Rob.	Craniotomy	Hospital	0.14	24	47.1	49.2	7.5	0	0	56.7	− 9.6	− 1.9
Lu.	Thyroidectomy	Hospital	0.15	25	46.6	45.5	2.0	0	0.1	47.6	− 1.0	− 0.2
Wo.	Craniotomy	Hospital	0.15	27	55.6	63.1	6.0	0	0	69.1	−13.5	− 2.7
Ham.	Expl. lapar.	Hospital	0.17	19	32.1	26.4	5.0	0	0	31.4	+ 0.7	+ 0.1
Fes.	Craniectomy	Glucose+hosp.	0.17	21	45.3	83.8	5.0*	0	0	90.8	−45.5	− 9.1
Wi.	Craniotomy	Hospital	0.17	29	34.3	33.7	1.5	0.2	0	35.4	− 1.1	− 0.2
Bu.	Craniotomy	P. K. gastrost.	0.18	19	77.9	120.0	5.9	0	0	125.9	−48.0	− 9.6
Brad.	Gastric resect.	Hospital	0.18	21	72.5	142.7	9.5	0	0	152.2	−79.7	−15.9
Ma.	Gastric resect.	Hospital	0.18	23	42.9	37.3	6.0	1.3	0	44.6	− 1.7	− 0.3
De.	Gastric resect.	Hospital	0.18	30	50.0	57.2	17.5	0.1	0	74.8	−24.8	− 4.9
War.	Expl. lapar.	Amigen+hosp.	0.19	25	53.0	57.0	7.0	1.3	0	65.3	−12.3	− 2.4
Ga.§	Gastric resect.	Amigen	0.19	22	57.1	44.6	16.5‡	3.4	0	64.5	− 7.4	− 1.6
Mc.	Gastro-enterost.	Amigen	0.20	22	54.3	51.2	16.5‡	6.6	1.6	75.9	−21.6	− 4.3
Mar.	Gastric resect.	Hospital	0.21	21	91.0	114.7	3.6	14.5	0	132.8	−41.8	− 8.3
Len.	Craniotomy	P. K. gastrost.	0.22	20	60.8	131.8	1.7	0	0	133.5	−62.7	−10.5
Fu.	Craniotomy	Gastrostomy mix.	0.22	31	51.0	72.2	25.0	0.7	0	97.9	−46.9	− 9.3
Th.T.	Craniotomy	Amigen+hosp.	0.24	31	77.6	99.2	5.5	1.4	0	106.1	−28.5	− 5.7
Be.	Craniotomy	Amigen	0.27	26	78.7	56.9	7.0†	0	1.4	65.3	+13.4	+ 2.6
Sc.	Ca. esophagus	Gastrostomy mix.	0.27	32	58.5	55.3	6.2	0	0	61.3	− 2.8	− 0.5
Po.	Gastrostomy	Gastrostomy mix.	0.28	31	64.5	56.2	16.5‡	0	0	72.7	− 8.2	− 1.6
Do.	Pit. explor.	Amigen+hosp.	0.30	30	109.5	101.9	4.0	0.3	0	106.2	+ 3.3	+ 0.6
Fo.	Gastric resect.	Lactalbumin	0.30	31	87.0	68.0	14.4	17.3	0	99.7	−12.7	− 2.5
Sm.E.	Craniotomy	Lactalbumin	0.30	32	99.0	113.0	7.0†	0	0	120.0	−21.0	− 4.2
Br.	Cranioplasty	Amigen+hosp.	0.30	33	88.2	70.1	7.0	0	0	77.1	+11.1	+ 2.2
Cl.	Gastric resect.	Amigen	0.30	33	75.0	55.5	9.5	4.6	0	69.6	+ 5.4	+ 1.0
Cu.	Craniotomy	Amigen	0.30	34	59.7	74.5	0.1	0.4	0	75.0	−15.3	− 3.0
Ga.	Craniotomy	Amigen	0.30	34	100.0	76.2	0.5	0	4.7	81.4	+18.6	+ 3.7
Ch.	Gastro-enterost.	Lactalbumin	0.30	36	70.5	41.1	7.5	5.5	0	54.1	+16.4	+ 3.2
Ba.	Gastric resect.	Lactalbumin	0.30	37	98.0	63.8	20.5	6.8	0	91.1	+10.4	+ 2.0
To.	Gastro-enterost.	Gastrostomy mix.	0.34	34	73.6	41.9	8.9	18.0	0	68.8	+ 4.8	+ 0.9
Ar.	Gastric resect.	Amigen	0.39	21	123.2	106.8	22.8	7.4	0.3	137.3	−14.1	− 2.9
Smi.	Gastrectomy	Gastrostomy mix.	0.39	32	98.8	57.5	30.0	4.5	0	92.0	+ 6.8	+ 1.3
Bra.	Gastrectomy	Amigen	0.46	32	137.8	65.1	10.0	55.5	0	130.6	+ 7.2	+ 1.4
St.	Gastro-enterost.	Amigen	0.46	35	117.6	102.5	9.0	8.0	0	119.5	− 1.9	− 0.3
Ro.	Expl. lapar.	Amigen+hosp.	0.47	46	106.0	78.2	6.0	0	0.2	84.4	+21.6	+ 4.3
No.	Craniotomy	Amigen	0.58	16	150.6	162.6	20.0	0	0	182.6	−32.6	− 6.5
Lo.	Gastric resect.	Amigen	0.59	11	180.0	163.3	25.0	6.5	0	194.8	−14.8	− 2.9
Bru.	Gastro-enterost.	Gastrostomy mix.	0.63	35	160.0	105.2	25.0	4.6	0	134.8	+25.2	+ 5.0

* Theoretical amount of 5.0 grams of nitrogen added to output for 5 days for fecal nitrogen not determined.
† Theoretical amount of 7.0 grams of nitrogen added to output for 5 days for fecal nitrogen not determined.
‡ Theoretical amount of 16.5 grams of nitrogen added to output for 5 days for fecal nitrogen not determined.
§ Jejunal feeding.

TABLE II

Fecal nitrogen

Type of feeding	No. of patients	Daily nitrogen output		
		Maximum	Minimum	Mean
		grams	*grams*	*grams*
By mouth— Hospital diet	18	1.5	0.1	1.0
By tube into stomach— Hydrolyzed protein or hydrolyzed protein plus hospital diet	12	25.0	0.1	1.4
By tube into jejunum— Hydrolyzed protein or whole protein as liquid feeding	13	25.0	6.2	3.3

trogen in the feces, and likewise the greatest variation in fecal nitrogen excretion was found in those fed by tube, into either the stomach or jejunum. The mean figures given in Table II were used in calculating balances in the cases where fecal nitrogen was not actually determined. The mean values for excretion of fecal nitrogen when food is given by mouth or by tube into the stomach agree with those given by Reifenstein, Albright and Wells (9).

Plasma proteins.

Because of the close relationship between hypoproteinemia and certain postoperative surgical complications (10 to 12), we were interested in studying the plasma protein concentration of surgical patients on various dietary regimes, but found that there was no significant correlation between the food intake and changes in plasma protein concentration, nor between nitrogen balance and changes in plasma protein concentration in this series of patients.

DISCUSSION

From the results obtained, it is apparent that surgical patients after operation require a larger intake of nitrogen to maintain positive nitrogen balance than has been recommended for normal individuals. Sherman (13) gives figures of 0.1 gram of nitrogen per kgm., and approximately 35 calories per kgm. for 1 normal individual. Rose and MacLeod (14) in a study of 4 normal active women found that 0.5 gram of protein (0.08 gram of nitrogen), and approximately 40 calories per kgm. daily, were sufficient. Mueller, Fickas and Cox (15), using an Amigen supplement to a basal diet, were able to produce considerable diminution in the amount of negative nitrogen balance in 4 normal men, and even to get positive balance in 2, giving 0.1 gram of nitrogen per kgm., and 26 to 28 calories per kgm. daily. The only nitrogen figures for normal individuals which approach the level found necessary by us for the immediate postoperative period are the amounts recommended by Howe (16) or the United States Army (men on moderate activity), 107.0 grams of protein and approximately 3,500 calories (for a 65 kgm. person this is 0.26 gram of nitrogen per kgm., and 53 calories per kgm. daily).

The increased nitrogen loss following trauma has been extensively studied in fractures of long bones (2, 3) and in burns (17, 18). Similar data for patients undergoing other types of surgical treatment, such as gastric or cranial operations, are meager. Ariel and his associates (19) have reported hypoproteinemia following operation for cancer of the gastro-intestinal tract. Elman (20) has frequently written of the poor state of nutrition of surgical patients, and reported on the use of hydrolyzed casein intravenously in the correction of hypoproteinemia in patients undergoing surgical operation. Mulholland, Co Tui *et al* (21) described the results obtained in 4 carefully studied postoperative gastric cases who received hydrolyzed casein as the source of nitrogen, together with 4 controls. Co Tui, Wright, Mulholland *et al* (22) reported studies on 19 postgastrectomy cases, of whom 8 on a high nitrogen, high caloric intake were in positive nitrogen balance. Brunschwig (23), in a series of 41 patients undergoing various types of operation, found that negative nitrogen balances were present in the majority of them. The dietary regimen of Brunschwig's patients was similar to that of many of our patients (Table II) and is the common one of intravenous fluids (usually saline or glucose solutions) followed by liquids by mouth on the third or fourth postoperative day, and soft to regular ward diet thereafter.

From these previously reported studies, it is not possible to determine the minimum level of nitrogen intake required to maintain the patient in nitrogen equilibrium. In our series 0.30 gram of nitrogen and 30 calories per kgm. daily proved to

be a level at which we could expect to have most patients in positive nitrogen balance in the early postoperative period. The relation between nitrogen intake and total calories was, in part, arbitrary. We recognize that it may have been possible to decrease the nitrogen level with a concurrent increase in caloric intake and still keep the patient in nitrogen equilibrium. While nitrogen intake has probably been more neglected in the management of surgical patients than caloric intake, the latter must not be allowed to fall too low. This is borne out by the data in Table I from 2 patients who had high nitrogen but low caloric intakes. It is interesting to note that in the cases reported by other workers (21, 22), where calculations can be made, and where the period of study is the same in relation to day of operation as in our studies, the amounts of nitrogen and the total calories with which they achieved positive nitrogen balance were well above the minimum effective level found in our studies.

It is clear from Table I that there are marked individual differences in the nitrogen excretion of patients on about the same nitrogen and caloric intake. Undoubtedly this is affected by anesthesia, the extent of operation, the degree of trauma, the previous nutritional history of the patient, the degree of postoperative pyrexia and many other factors. It, however, seemed useful to determine approximately the amount of food required to keep the majority of patients in balance after gastric and intracranial operations.

A criterion frequently used as a measure of the effectiveness of procedures intended to correct protein deficiencies in surgical patients is the concentration of plasma or serum protein. Our results indicate that plasma protein concentration may be misleading as an index of whether or not the patient is overcoming excessive nitrogen loss. In some instances, although the concentrations (corrected for change in hematocrit) were increased, the patients were in negative nitrogen balance.

In this study, hydrolyzed protein was found to be just as effective as whole protein in maintaining nitrogen balance.

A real difficulty in the forced feeding of patients in the early postoperative period is the frequency with which vomiting, diarrhea or distention follows such a program. Usually it was possible to overcome these obstacles by symptomatic treatment, but occasionally it was necessary to abandon the intended regimen. It is quite possible that intravenous feeding, rather than mouth feeding, may have a place of special importance in maintaining the nutrition of the patient in the first 48 to 72 hours after operation.

It should be emphasized that this study is concerned mainly with types of patients which are apt to present nutritional problems before and after operation. It is recognized that such patients represent a small minority of all general surgical patients, and it is not the intention of the authors to suggest that the forced feeding methods described are indicated for the majority of surgical patients.

CONCLUSIONS

1. Surgical patients after gastric or cranial operations were usually in negative nitrogen balance for the first 5 postoperative days on the dietary regimen customarily followed in this clinic.
2. Surgical patients undergoing gastric or cranial operations require a greater intake of dietary nitrogen and of total calories than do normal individuals to keep them in nitrogen equilibrium for the first 5 postoperative days.
3. By increasing the nitrogen intake to 0.30 gram per kgm., and the caloric intake to 30 calories per kgm. daily, or over, patients fed by mouth or Abbott-Rawson tube were usually maintained in nitrogen equilibrium.
4. Hydrolyzed protein in the form of a casein hydrolysate, combined with carbohydrate, was equally as effective as whole protein when administered by the same routes.
5. The variations in fecal nitrogen were large, and they are an important part of the nitrogen balance study of patients in the early postoperative period, especially when tube feedings are used.
6. The plasma or serum protein concentrations were not an accurate index of the effect of a nutritional regimen on nitrogen equilibrium in postoperative patients.

BIBLIOGRAPHY

1. Lund, C. C., and Levenson, S. M., Protein in surgery. J. A. M. A., 1945, 128, 95.
2. Cuthbertson, D. P., Further observations on the disturbance of metabolism caused by injury, with par-

ticular reference to the dietary requirements of fracture cases. Brit. J. Surg., 1935–1936, 23, 505.

3. Howard, J. E., Parson, W., Stein, K. E., Eisenberg, H., and Reidt, E. V., Studies on fracture convalescence; nitrogen metabolism after fracture and skeletal operations in healthy males. Bull. Johns Hopkins Hosp., 1944, 75, 156.
4. Stengel, A., Jr., and Ravdin, I. S., Maintenance of nutrition in surgical patients, with description of orojejunal method of feeding. Surgery, 1939, 6, 511.
5. Bisgard, J., Gastrostomy. Jejunal intubation. Surg., Gynec. and Obst., 1942, 74, 239.

6a. Chibnall, A. C., Rees, M. W., and Williams, E. F., Total nitrogen content of egg albumin and other proteins. Biochem. J., 1943, 37, 354.

6b. Meeker, E. W., and Wagner, E. C., Titration of ammonia in presence of boric acid. J. Ind. Eng. Chem. Anal. Ed., 1933, 5, 396. 1940, 12, 771.

7. Koch, F. C., and McMeekin, T. L., A new direct nesslerization micro Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Am. Chem. Soc., 1924, 46, 2066.

8a. Sherman, H. C., and Lanford, C. S., Essentials of Nutrition. Macmillan Co.

8b. Cooper, L. F., Barbour, E. M., and Mitchell, A. F., Nutrition in Health and Disease. J. B. Lippincott Co.

9. Reifenstein, E. C., Jr., Albright, F., and Wells, S. L., Accumulation, interpretation and presentation of data pertaining to metabolic balances, notably those of calcium, phosphorus, and nitrogen. J. Clin. Endocrinol., 1945, 5, 367.
10. Jones, C. M., and Eaton, F. B., Postoperative nutritional edema. Arch. Surg., 1933, 27, 159.
11. Mecray, P. M., Barden, R. P., and Ravdin, I. S., Nutritional edema. Its effect on gastric emptying time before and after gastric operations. Surgery, 1937, 1, 53.
12. Thompson, W. C., Ravdin, I. S., and Frank, I. L., Effect of hypoproteinemia on wound disruption. Arch. Surg., 1938, 36, 500.
13. Sherman, H. C., Gillett, L. H., and Osterberg, E., Protein requirement of maintenance in man and the nutritive efficiency of bread protein. J. Biol. Chem., 1920, 41, 97.
14. Rose, M. S., and Macleod, G., Maintenance values for proteins of milk, meat, bread and milk, and soy bean curd. J. Biol. Chem., 1925, 66, 847.
15. Mueller, A. J., Fickas, D., and Cox, W. M., Minimum maintenance requirement of an enzymic casein hydrolysate. Bull. Johns Hopkins Hosp., 1943, 72, 110.
16. Howe, P. E., Nutrition as it affects military personnel. Med. Clin. North America, 1943, 27, 581.
17. Taylor, F. H. L., Levenson, S. M., Davidson, C. S., Browder, N. C., and Lund, C. C., Problems of protein nutrition in burned patients. Ann. Surg., 1943, 118, 215.
18. Hirshfeld, J. W., Abbott, W. E., Pilling, M. A., Heller, C. G., Meyer, F., Williams, H. H., Richards, A. J., and Obi, R., Metabolic alterations following thermal burns. III. Effect of variations in food intake on nitrogen balance of burned patients. Arch. Surg., 1945, 50, 194.
19. Ariel, I., Abels, J. C., Pack, G. T., and Rhoads, C. P., Postoperative hypoproteinemia and relationship of serum protein fall to urinary nitrogen excretion. Surg., Gynec. and Obst., 1943, 77, 16.
20. Elman, R., Oral use of amino acids of hydrolysed casein in surgical patients. Am. J. Digest. Dis., 1943, 10, 48.
21. Mulholland, J. H., Co Tui, F. W., Wright, A. M., and Vinci, V. J., Nitrogen metabolism, caloric intake, and weight loss in postoperative convalescence. A study of eight patients undergoing partial gastrectomy for duodenal ulcers. Ann. Surg., 1943, 117, 512.
22. Co Tui, F. W., Wright, A. M., Mulholland, J. H., Carabba, V., Barcham, I., and Vinci, V. J., Studies on surgical convalescence. I. Sources of nitrogen loss postgastrectomy and effect of high amino acid and high caloric intake on convalescence. Ann. Surg., 1944, 120, 99.
23. Brunschwig, A., Clark, D. E., and Corbin, N., Postoperative nitrogen loss and studies on parenteral nitrogen nutrition by means of casein digest. Ann. Surg., 1942, 115, 1091.

THE METABOLIC EFFECTS OF STEROID HORMONES IN OSTEOPOROSIS

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In a previous communication from this clinic (1), three metabolic studies on the effect of estradiol benzoate on the calcium and phosphorus metabolisms of patients with post-menopausal osteoporosis were published in abstract form. The first objective of the present paper is to report these studies in detail, supplemented by 2 additional studies: one in which testosterone propionate by itself, and in combination with estradiol benzoate, was used; and another in which diethylstilbestrol by itself, and in combination with progesterone, was employed. The subject of the last investigation had, in addition to post-menopausal osteoporosis, Paget's disease.

The second objective is to publish metabolic studies on the effect of testosterone propionate alone and in combination with estradiol benzoate in a male patient with senile osteoporosis.

The third objective is to present studies on 3 patients with the acute osteoporotic process which follows orthopedic operations, and the effect of estradiol benzoate on this process in 2 of these subjects.

In another previous communication from this clinic (2), metabolic studies of the effect of estradiol benzoate, testosterone propionate, and progesterone on 3 patients with Cushing's syndrome were reported. The fourth objective is to present these data more completely in graphic form, and especially to rectify an unwarranted conclusion as to the effect of estrogen on the calcium balance.

DEFINITION OF OSTEOPOROSIS

Osteoporosis is not synonymous with demineralization of bone; it is that category of too-little-bone where the primary disturbance is lack of bone matrix formation. It is not to be confused with osteomalacia, where the primary disturbance is failure of mineralization of bone, or with osteitis fibrosa generalisata, where the primary disturbance is increased bone destruction. For further discussion, see (1, 3, 4).

CONDITIONS ASSOCIATED WITH OSTEOPOROSIS

In clinical medicine one encounters the following conditions associated with osteoporosis: (*1*) disuse atrophy, where the normal stimulus to osteoblastic activity is absent (4, 5); (*2*) old age, where the bone tissue like other tissue (*cf.* hair, skin, muscles) atrophies; (*3*) malnutrition, where the protein requirements are not fulfilled, and the bone matrix, like other tissues, is depleted; (*4*)

[1] The expense of these studies was defrayed by grants from the Josiah Macy, Jr. Foundation, from the Rockefeller Foundation, and from the National Research Council (Committee for Research in the Problems of Sex). A bed supported by the Mallinckrodt Chemical Company on the Metabolic Ward was used for part of these studies.

[2] Presented in part at the twenty-sixth annual meeting of the Association for the Study of Internal Secretions, Atlantic City, New Jersey, June 8, 1942, in connection with a symposium on "Relation of Endocrines to Skeletal Development"; an outline of this presentation may be found in: Reifenstein, E. C., Jr.; Albright, F.; Parson, W.; and Bloomberg, E.: The effect of estradiol benzoate and of testosterone propionate and of combinations of both on post-menopausal osteoporosis and senile osteoporosis, Endocrinology, 30: S1024 (1942). Also presented in part at the first annual meeting of the American Federation for Clinical Research, Minneapolis, Minn., April 20, 1942. Preliminary reports of part of these data may be found in: Albright, F.; Reifenstein, E. C., Jr.; and Forbes, A. P.: Conferences on the Metabolic Aspects of Convalescence (Including Bone and Wound Healing), Transactions of the First Meeting, Sept. 11–12, 1942, pages 5–7, 37–38; Transactions of the Second Meeting, December 11–12, 1942, pages 69, 96–98; Transactions of the Third Meeting, March 12–13, 1943, pages 63–65; and Transactions of the Fourth Meeting, June 11–12, 1943, pages 77–85. Transactions distributed by the Josiah Macy, Jr. Foundation, New York, N. Y.

[3] The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital.

TABLE I

Data for case 1 (F.F., M.G.H. 156453)

Period number	Date	Calcium: Urinary	Calcium: Fecal	Calcium: Intake	Calcium: Balance	Phosphorus: Urinary	Phosphorus: Fecal	Phosphorus: Intake	Phosphorus: Balance	Nitrogen: Urinary	Nitrogen: Intake	Nitrogen: Balance	Nitrogen: Theoretical balance	Body weight: Measured*	Body weight: Theoretical	Serum: Day of period	Serum: Calcium	Serum: Phosphorus	Serum: Alkaline phosphatase	Treatment: Estradiol benzoate (i.m.)	Treatment: Estradiol dipropionate (i.m.)	Treatment: Progesterone (i.m.)
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*				*kgm.*			*mgm. per 100 ml.*		*B.U.*			
1	11/14 to 18/38	289	620	735	−174	371	288	606	− 53	6.99	7.69	−0.07	+0.51	51.03	51.12	I	9.7	4.0		None	None	None
2	11/19 to 23/38	286	514	735	− 65	386	225	606	− 5	6.80	7.69	+0.12	+0.43	51.13	51.13							
3	11/24 to 28/38	278	412	735	+ 45	364	171	606	+ 71	6.74	7.69	+0.18	+0.72	51.13	51.15							
4	11/29 to 12/2/38	259	455	735	+ 21	312	235	606	+ 59	6.60	7.69	+0.32	+0.72	51.37	51.18	I	9.3	4.5	3.3	1.66 mgm. every third day		
5	12/ 3 to 7/38	249	349	735	+137	237	170	606	+199	6.14	7.69	+0.78	+1.95	51.47	51.28	III	9.4	3.8				
6	12/ 8 to 12/38	210	486	735	+ 39	276	268	606	+ 62	5.95	7.69	+0.97	+0.65	51.74	51.41							
7	12/13 to 17/38	187	323	735	+225	262	194	606	+150	6.39	7.69	+0.53	+0.55	51.90	51.48	II	9.7	4.4				
8	12/18 to 22/38	182	527	735	+ 26	226	282	606	+ 98	6.14	7.69	+0.78	+1.27	51.97	51.58							
	No collections for 23 days													51.97	51.58							
9	1/16 to 20/39	180	395	735	+160	217	229	606	+160	6.18	7.69	+0.74	+1.20	52.23	51.67	I	9.4	3.6	2.3			
10	1/21 to 25/39	161	430	735	+141	226	254	606	+126	5.95	7.69	+0.97	+0.81	52.28	51.80							10 mgm. daily
11	1/26 to 30/39	178	418	735	+139	213	237	606	+156	6.93	7.69	−0.01	+1.31	52.06	51.79	I	10.4	4.4	3.1			10 mgm. daily
12	1/31 to 2/4/39	149	418	735	+168	227	259	606	+120	6.38	7.69	+0.54	+0.54	52.68	51.86	V	9.0	3.7	3.4			
13	2/ 5 to 9/39	181	615	735	− 61	156	315	606	+135	5.62	7.69	+1.30	+2.49	53.40	52.03					None		None
14	2/10 to 14/39	147	198	735	+390	258	113	606	+235	5.97	7.69	+0.95	+0.60	52.47	52.16							
15	2/15 to 19/39	138	432	735	+165	288	245	606	+ 73	7.33	7.69	−0.41	−0.15	52.04	52.09							
16	2/20 to 24/39	163	472	735	+100	269	263	606	+ 74	7.06	7.69	−0.14	+0.36	52.42	52.06	I	10.0	3.9	3.4			
17	2/25 to 3/1/39	153	431	735	+151	269	244	606	+ 93	6.52	7.69	+0.40	+0.25	52.40	52.11							
18	3/ 2 to 6/39	171	717	735	−153	298	378	606	− 70	6.59	7.69	+0.33	+0.11	52.19	52.15							
19	3/ 7 to 11/39	192	295	735	+248	286	158	606	+162	6.01	7.69	+0.91	+0.57	52.36	52.27	I	9.9	4.5	2.1			
20	3/12 to 16/39	207	555	735	− 27	293	258	606	+ 55	6.17	7.69	+0.75	+1.03	52.09	52.37							
21	3/17 to 21/39	219	695	735	−179	296	296	606	+ 14	6.21	7.69	+0.71	+1.55	52.37	52.46	IV	10.0	4.4	2.8			
22	3/22 to 26/39	234	485	735	+ 16	309	210	606	+ 87	6.86	7.69	+0.06	+1.19	52.31	52.46	I	9.6	4.7	3.1			
23	3/27 to 31/39	237	610	735	−112	292	260	606	+ 54	6.61	7.69	+0.31	+1.65	52.77	52.49	I	10.7	4.6	2.5			
24	4/ 1 to 5/39	231	677	735	−173	207	260	606	+139	6.07	7.69	+0.85	+3.39	52.60	52.60	V	9.8	4.6	3.0			
	No collections for 79 days													51.85						1.66 mgm. every third day		
25	6/25 to 29/39	197	417	735	+121	256	207	606	+143	6.22	7.69	+0.70	+1.17	52.19						1.66 mgm. every third day		
	No collections for 297 days													49.60						1.66 mgm. every fifth day		
26	4/22 to 26/40	226	692	735	−183	246	345	606	+ 15	8.13	7.69	−1.21	+1.61	49.70						1.66 mgm. every third day		
27	4/27 to 5/1/40	207	865	735	−337	287	392	606	− 73	6.46	7.69	+0.46	+2.22	49.60						1.66 mgm. every third day		
	No collections for 602 days													47.13		12/23/41	10.0	3.8	2.0	Pellets 195 mgm.	5 mgm. every 10 days**	
28	12/25 to 29/41	216	441	735	+ 78	231	219	606	+156	6.39	7.69	+0.53	+1.77	48.33						1.66 mgm. every third day	None	
29	12/30 to 1/3/42	244	513	735	− 22	245	272	606	+ 89	6.37	7.69	+0.55	+1.51	48.27						1.66 mgm. every third day	None	
30	1/ 4 to 8/42	256	513	735	− 34	273	272	606	+ 61	6.56	7.69	+0.36	+1.19	48.07						1.66 mgm. every third day	None	
	1/9/42																8.5	4.1	1.7			

Dietary intake of periods 1 to 30 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 48.1 grams, fat (estimated from tables) = 85.0 grams, carbohydrate (estimated from tables) = 212.8 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat, and 4 for 1 gram of carbohydrate) = 1,809. In addition sugar was given *ad lib*, with an average intake of 30 grams (120 calories).

* Initial weight 51.14 kgm.

** Continued for 6 months.

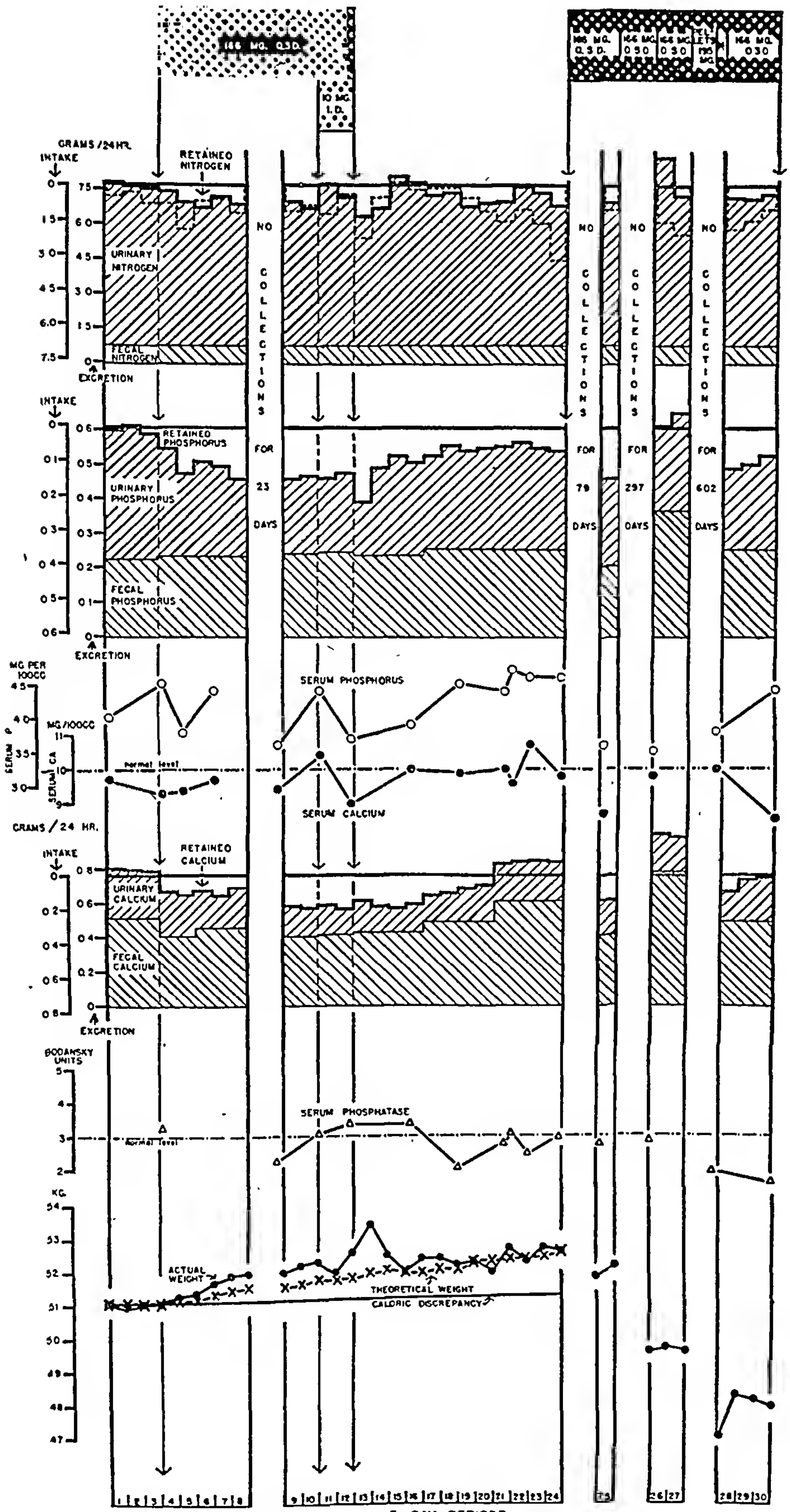

FIG. 1. CASE 1 (F. F., M.G.H. 156453): EFFECT OF ESTRADIOL BENZOATE ON NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES, ON SERUM

Cushing's syndrome where, we believe, an excess of the adrenal cortical "sugar" or "S" hormone inhibits anabolism of protoplasm including bone matrix (2, 6); (5) adaptation syndrome of Selye (7), where, we believe, the pathological physiology is the same as in Cushing's syndrome; (6) idiopathic osteoporosis, where the cause of the condition remains obscure; (7) acromegaly, where the cause may be the increase of pituitary hormone(s), or the secondary lack of gonadal hormones (8); and (8) the post-menopausal state, the commonest of all forms, where the difficulty is a deficiency in estrogen to stimulate the osteoblasts. Frequently 2 or more factors combine in one individual; thus, after an orthopedic operation (see Cases 7, 8, and 9, below) factors (*1*) and (*5*) probably both play a part.

METABOLIC STUDIES

For the methods employed in the accumulation, interpretation and presentation of these data, see (9). Case histories are abstracted in the appendix.

A. Post-menopausal osteoporosis

Case 1. Post-Menopausal Osteoporosis; Artificial Menopause; Estradiol Benzoate Therapy.

The metabolic data of Case 1 are shown in Figure 1 and Table I. The first part of the study, conducted in 5-day periods, consisted of: (*1*) three control periods; (*2*) five periods with estradiol benzoate 1.66 mgm. intramuscularly every 3 days; (*3*) twenty-three days with the same therapy at home; (*4*) two periods with the same therapy; (*5*) two periods with progesterone 10 mgm. intramuscularly daily in addition to the estradiol; and (*6*) twelve periods after the cessation of both medications. The patient was then discharged on estrogen therapy which was given continuously in varied dosage during the next 3 years; during this interval she was brought back to the metabolic ward for study (1 to 3 five-day periods) on 3 occasions.

The data (Figure 1) are self-explanatory. Attention should be called to: (*1*) nitrogen, phosphorus, and calcium equilibria during the control periods (1 to 3); (*2*) the high serum phosphorus level which tended to fall under estrogen therapy (less marked in this case than in the others [*vide infra*]); (*3*) the slight improvement in nitrogen balance under estrogen therapy; (*4*) the striking and growing decrease in calcium excretion, both fecal and urinary, with estrogen treatment and the gradual return (40 days) in calcium excretion to pre-treatment levels following cessation of estrogen therapy; (*5*) a decrease with estrogen treatment in the phosphorus excretion almost entirely confined to the urinary component, and reasonably proportional to the changes in the calcium and nitrogen metabolisms (see "Theoretical Nitrogen Balance"); (*6*) failure of the serum phosphatase level, the index of osteoblastic activity, to rise under estrogen therapy; (*7*) an increase in nitrogen, but not in calcium and phosphorus, excretions in periods 11 and 12 with progesterone therapy; and (*8*) the tendency to retain extracellular fluids with estradiol therapy, as suggested by the increase in the actual weight above the theoretical weight.

The apparent discrepancy in the effect of estrogen on the calcium and phosphorus balances during periods 26 and 27 is probably to be explained by erroneously high fecal excretions resulting from too short a period of observation (9).

Case 2. Post-Menopausal Osteoporosis; Physiological Menopause; Question of Superimposed Atrophy of Disuse; Estradiol Benzoate Therapy.

The metabolic data of Case 2 are shown in Figure 2 and Table II. The study, conducted in 5-day periods, consisted of: (*1*) five control periods; (*2*) thirteen periods during which the patient received estradiol benzoate 3.32 mgm. intramuscularly every other day. In addition, during the 3 periods 14, 15, and 16, testosterone propionate 25 mgm. were administered intramuscularly every other day.

The data in Case 2 confirm the main observations made on Case 1. The fall in the serum phosphorus level after estradiol medication was more pronounced than in Case 1, and in addition there was a fall in the serum calcium level. Again the serum phosphatase level failed to rise with the improvement in the calcium balance. The duration of the testosterone propionate therapy was too short to judge its effect on the calcium balance; it brought about the expected increase in the nitrogen retention and rise in the urinary 17-ketosteroid excretion. The theoretical nitrogen balance based on the phosphorus balance after it had been corrected for the calcium balance agrees quite well with the measured nitrogen balance.

CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS, AND ON BODY WEIGHT IN A FEMALE PATIENT WITH POST-MENOPAUSAL OSTEOPOROSIS

For discussion, see text.

The dotted line in the nitrogen metabolism data represents the "theoretical nitrogen balance." The fecal nitrogen was estimated as 10 per cent of the intake. The fecal calcium and phosphorus values as charted are averages of 1, 2, 3, or 4 five-day periods as follows: 1 through 3, 4 through 5, 6 through 8, 9 through 10, 11 through 12, 13 through 16, 17 through 20, 21 through 24, 25, 26 through 27, 28 through 30; the individual values are given in Table I.

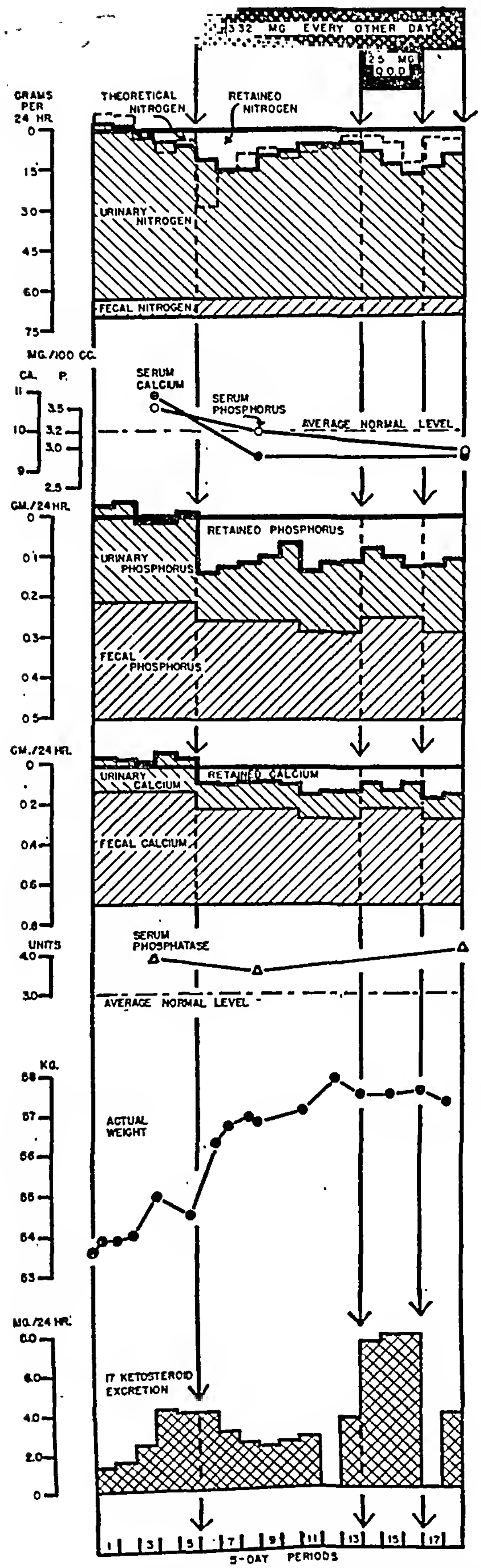

FIG. 2. CASE 2 (E. P., M.G.H. 203540): EFFECT OF ESTRADIOL BENZOATE AND TESTOSTERONE PROPIONATE ON

Case 3. Post-Menopausal Osteoporosis; Artificial Menopause; Estradiol Benzoate Therapy.

The metabolic data of Case 3 are shown in Figure 3 and Table III. The study, conducted in 5-day periods, consisted of: (*1*) four control periods; (*2*) nine periods in which 1.66 mgm. of estradiol benzoate were administered intramuscularly every 3 days; (*3*) ninety-three days at home on the same medication; (*4*) five periods on the same medication; (*5*) seven periods during which the estradiol dosage was doubled; and (*6*) five control periods of medication. During period 10 the patient was given in addition 10 mgm. of progesterone intramuscularly each day.

It will be noted in Figure 3 that the improvement in the calcium balance in this case following estradiol therapy was almost entirely due to the fall in the urinary calcium excretion. It is further suggested that the positive calcium balance tends to diminish with time (compare periods 14 to 18 with periods 11 to 13). Note, furthermore, that the calcium balance was not improved, and possibly reduced, when estradiol therapy was doubled in periods 19 through 25. The fall in the serum phosphorus level with medication was especially striking in this case. The actual weight was greater than the theoretical weight during the therapy, which suggests retention of extracellular fluids.

Case 4. Post-Menopausal Osteoporosis; Artificial Menopause; Methyl Testosterone, Estradiol Benzoate and Pregnenolone Therapy.

The metabolic data of Case 4 are given in Figure 4 and Table IV. The study, conducted in 6-day periods, consisted of: (*1*) four control periods; (*2*) four periods on methyl testosterone, 40 mgm. by mouth daily; (*3*) five periods in which 1.66 mgm. of estradiol benzoate daily by injection were added to the methyl testosterone therapy; (*4*) five periods back on the methyl testosterone therapy alone; (*5*) four more control periods off medication; (*6*) three periods on pregnenolone, 30 mgm. intramuscularly daily; (*7*) four more control periods off medication; (*8*) five periods back on methyl testosterone, 40 mgm. by mouth daily with a change in the nitrogen and phosphorus intakes during the last 3 of these; and (*9*) one final period where the methyl testosterone therapy was increased to 100 mgm. by mouth daily. The urinary determinations were made on 3-day periods throughout.

In Figure 4 it should be noted first that the theoretical nitrogen balance is consistently less than the actual

NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES, ON SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS, ON BODY WEIGHT AND ON URINARY 17-KETOSTEROID EXCRETION

For discussion, see text.

The fecal nitrogen was estimated as 10 per cent of the intake. The fecal phosphorus and calcium values as charted are averages of 2, 3, 4, or 5 five-day periods as follows: 1 through 5, 6 through 9, 10 through 13, 14 through 16, 17 through 18; the individual values are given in Table II.

TABLE II

Data for case 2 (E.P., M.G.H. 203540)

Period number	Date	Calcium				Phosphorus				Nitrogen				Body weight		Urinary 17-keto-steroids		Serum				Treatment	
		Urinary	Fecal	Intake	Balance	Urinary	Fecal	Intake	Balance	Urinary	Intake	Balance	Theoretical balance	Measured*	Theoretical	Day of period		Day of period	Calcium	Phosphorus	Alkaline phosphatase	Estradiol benzoate (i.m.)	Testosterone propionate (i.m.)
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*				*kgm.*			*mgm. per 24 hr.*		*mgm. per 100 ml.*		*B.U.*		
	9/18/39															IV	1.5		10.1	3.5	3.7	None	None
1	9/23 to 27/39	183	428	708	+ 97	238	256	506	+ 12	6.47	6.93	−0.23	−0.57	53.90	53.71	V	2.4						
2	9/28 to 10/2/39	177	594	708	− 63	255	314	506	− 63	6.39	6.93	−0.15	−0.48	53.86	53.77								
3	10/ 3 to 7/39	146	624	708	− 62	198	329	506	− 21	5.92	6.93	+0.32	+0.15	54.02	53.90			II	10.9	3.5	3.9		
4	10/ 8 to 12/39	187	581	708	− 60	197	284	506	+ 25	5.78	6.93	+0.46	+0.79	54.96	54.06	II	4.2						
5	10/13 to 17/39	162	589	708	− 43	223	278	506	+ 5	5.64	6.93	+0.60	+0.37	54.48	54.23	IV	4.0						
6	10/18 to 22/39	133	377	708	+198	118	193	506	+195	5.12	6.93	+1.12	+2.95	56.38	54.48	IV	4.0					3.32 mgm. every other day	
7	10/23 to 27/39	129	671	708	− 92	127	322	506	+ 57	4.74	6.93	+1.50	+1.51	56.70	54.79	IV	3.0	V	9.4	3.2	3.5		
8	10/28 to 11/1/39	139	471	708	+ 98	146	249	506	+111	4.72	6.93	+1.52	+0.91	56.83	55.10	IV	2.4						
9	11/ 2 to 6/39	142	404	708	+162	160	215	506	+131	5.27	6.93	+0.97	+0.73		55.34	IV	2.2						
10	11/ 7 to 11/39	131	407	708	+170	195	178	506	+133	5.45	6.93	+0.79	+1.21		55.54	II	2.4						
11	11/12 to 16/39	125	427	708	+156	162	238	506	+106	5.69	6.93	+0.55	+0.71	57.11	55.70	IV	2.6						
12	11/17 to 21/39	136	471	708	+101	175	236	506	+ 95	5.71	6.93	+0.53	+0.65	57.89	55.86								
13	11/22 to 26/39	141	431	708	+136	178	244	506	+ 84	5.73	6.93	+0.51	+0.23		56.02	IV	3.6						
14	11/27 to 12/1/39	127	481	708	+100	173	267	506	+ 66	5.47	6.93	+0.77	+0.21	57.49	56.22	V	7.5						25 mgm. every other day
15	12/ 2 to 6/39	91	367	708	+250	151	205	506	+150	4.95	6.93	+1.29	+0.51	57.52	56.50	IV	7.8						25 mgm. every other day
16	12/ 7 to 11/39	122	595	708	− 9	129	297	506	+ 80	4.61	6.93	+1.63	+1.25	57.64	56.81	IV	7.8						25 mgm. every other day
17	12/12 to 16/39	106	407	708	+195	168	202	506	+136	4.79	6.93	+1.45	+0.39	57.34	57.11								None
18	12/17 to 21/39	124	446	708	+138	185	222	506	+ 99	5.24	6.93	+1.00	+0.43	57.34	57.34	II	3.8						None
	12/22/39																		9.4	2.9	4.1		

Dietary intake of periods 1 to 18 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 43.3 grams, fat (estimated from tables) = 75.8 grams, carbohydrate (estimated from tables) = 213.3 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat, and 4 for 1 gram of carbohydrate) = 1,609. In addition sugar was given *ad lib*, with an average intake of 30 grams (120 calories).

* Initial weight (9/23/39) 53.66 kgm.

** Urinary 17-ketosteroid on 9/20/39 1.3 mgm. per 24 hours.

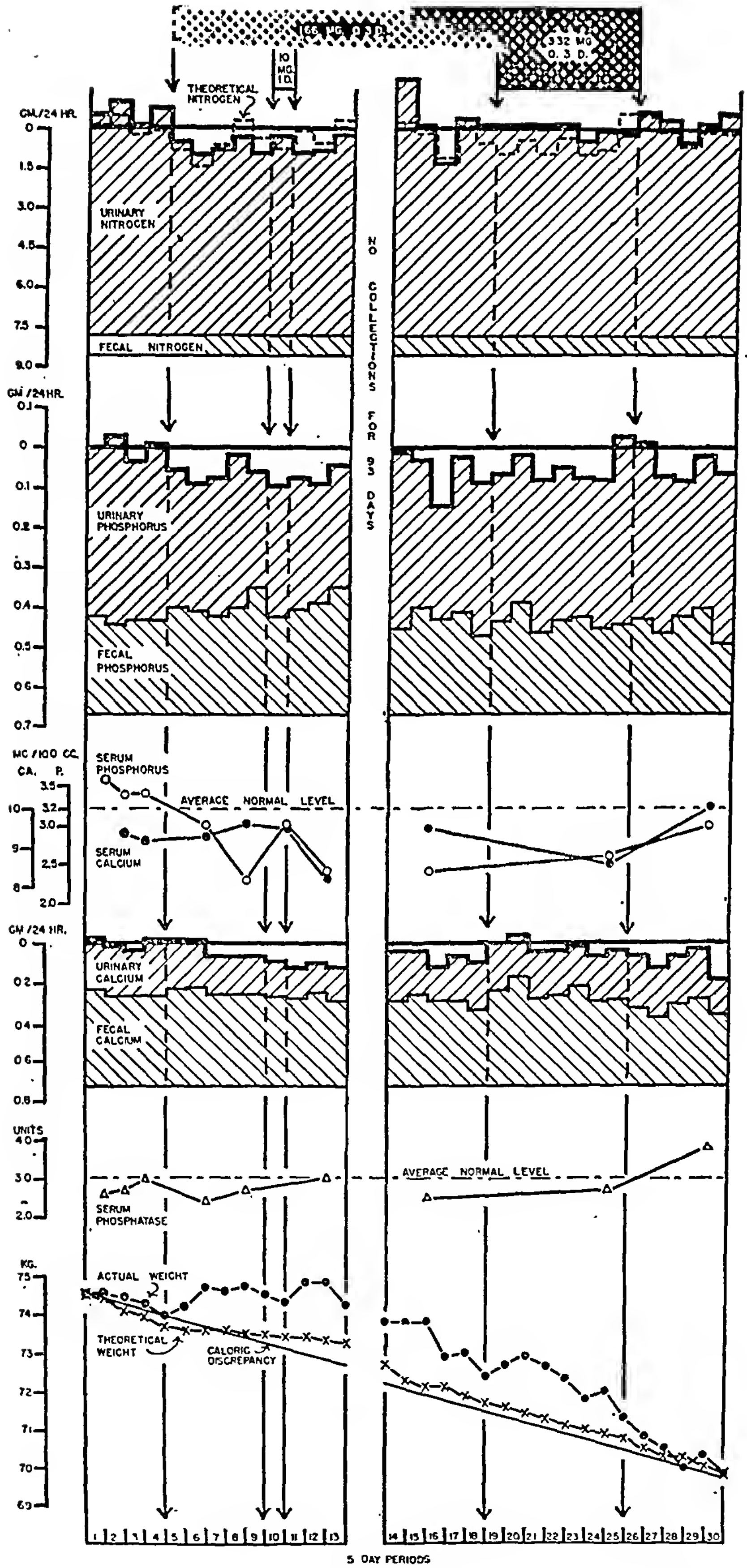

FIG. 3. CASE 3 (A. M. R., M.G.H. 29358): EFFECT OF ESTRADIOL BENZOATE ON NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES, ON SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS, AND ON BODY WEIGHT IN A FEMALE PATIENT WITH POST-MENOPAUSAL OSTEOPOROSIS

For discussion, see text.

TABLE III

Data for case 3 (A.M.R., M.G.H. 29358)

Period number	Date	Calcium: Urinary	Calcium: Fecal	Calcium: Intake	Calcium: Balance	Phosphorus: Urinary	Phosphorus: Fecal	Phosphorus: Intake	Phosphorus: Balance	Nitrogen: Urinary	Nitrogen: Intake	Nitrogen: Balance	Nitrogen: Theoretical balance	Body weight: Measured*	Body weight: Theoretical	Serum: Day of period	Serum: Calcium	Serum: Phosphorus	Serum: Alkaline phosphatase	Treatment: Estradiol benzoate (i.m.)	Treatment: Progesterone (i.m.)
		mgm. per 24 hr.				mgm. per 24 hr.				grams per 24 hr.				kgm.			mgm. per 100 ml.		B.U.		
1	4/23 to 27/39	251	505	739	− 17	412	255	667	0	8.21	8.56	−0.51	−0.13	74.53	74.50					None	None
2	4/28 to 5/2/39	276	460	739	+ 3	467	234	667	− 34	8.69	8.56	−0.99	−0.53	74.37	74.27	I		3.6	2.5		
3	5/ 3 to 7/39	251	460	739	+ 28	389	246	667	+ 32	7.72	8.56	−0.02	+0.27	74.28	74.21	I	9.4	3.4	2.6		
4	5/ 8 to 12/39	292	460	739	− 13	428	244	667	− 5	8.44	8.56	−0.74	+0.03	74.00	74.03	I	9.2	3.4	2.9		
5	5/13 to 17/39	246	499	739	− 6	342	274	667	+ 51	7.14	8.56	+0.56	+0.81	74.23	74.01					1.66 kgm. every third day	
6	5/18 to 22/39	251	510	739	− 22	313	267	667	+ 87	6.77	8.56	+0.93	+1.17	74.76	74.10						
7	5/23 to 27/39	201	475	739	+ 63	343	251	667	+ 73	6.91	8.56	+0.79	+0.63	74.66	74.15	I	9.3	3.0	2.3		
8	5/28 to 6/1/39	199	477	739	+ 63	385	270	667	+ 12	7.32	8.56	+0.38	−0.29	74.76	74.13						
9	6/ 2 to 6/39	193	479	739	+ 67	294	319	667	+ 54	6.80	8.56	+0.90	+0.31	74.55	74.19	I	9.6	2.3	2.6		
10	6/ 7 to 11/39	187	466	739	+ 86	320	253	667	+ 94	7.38	8.56	+0.32	+0.77	74.34	74.16						10 mgm. daily
11	6/12 to 16/39	151	455	739	+133	328	267	667	+ 72	6.83	8.56	+0.87	+0.09	74.80	74.22	I	9.5	3.0			None
12	6/17 to 21/39	163	481	739	+ 95	303	279	667	+ 85	6.92	8.56	+0.78	+0.57	74.81	74.26						
13	6/22 to 26/39	181	438	739	+120	308	321	667	+ 38	7.41	8.56	+0.29	−0.33	74.23		I	8.3	2.4	2.9		
	No collections for 93 days													73.73	73.73						
14	9/23 to 27/39	263	437	739	+ 39	447	219	667	+ 1	9.64	8.56	−1.94	−0.27	73.68	73.23						
15	9/28 to 10/2/39	231	478	739	+ 30	372	268	667	+ 27	7.80	8.56	−0.10	+0.18	73.73	73.01						
16	10/ 3 to 7/39	173	431	739	+135	277	245	667	+145	6.50	8.56	+1.20	+1.17	72.94	72.98	I	9.5	2.4	2.4		
17	10/ 8 to 12/39	254	428	739	+ 57	385	263	667	+ 19	8.10	8.56	−0.40	−0.15	72.98	72.71						
18	10/13 to 17/39	255	387	739	+ 97	376	206	667	+ 85	7.76	8.56	−0.06	+0.54	72.37	72.49						
19	10/18 to 22/39	246	493	739	0	371	237	667	+ 59	7.85	8.56	−0.15	+0.89	72.67	72.26					3.32 mgm. every third day	
20	10/23 to 27/39	214	564	739	− 39	375	278	667	+ 14	7.80	8.56	−0.10	+0.49	72.95	72.04						
21	10/28 to 11/1/39	253	451	739	+ 35	378	215	667	+ 74	7.86	8.56	−0.16	+0.85	72.62	71.81						
22	11/ 2 to 6/39	238	465	739	+ 36	382	242	667	+ 43	7.81	8.56	−0.11	+0.37	72.27	71.58						
23	11/ 7 to 11/39	219	513	739	+ 7	346	253	667	+ 68	7.22	8.56	+0.48	+0.97	71.88	71.44						
24	11/12 to 16/39	247	433	739	+ 59	370	220	667	+ 77	7.68	8.56	+0.02	+0.72	72.06	71.24						
25	11/17 to 21/39	266	446	739	+ 27	466	231	667	− 30	7.50	8.56	+0.20	−0.65	71.28	71.06	I	8.6	2.6	2.6		
26	11/22 to 26/39	277	401	739	+ 61	432	245	667	− 10	8.36	8.56	−0.66	−0.61	70.80	70.75					None	
27	11/27 to 12/1/39	263	357	739	+119	389	213	667	+ 65	8.13	8.56	−0.43	+0.09	70.54	70.48						
28	12/ 2 to 6/39	256	428	739	+ 55	338	250	667	+ 79	7.11	8.56	+0.59	+0.63	69.98	70.36						
29	12/ 7 to 11/39	261	455	739	+ 23	384	269	667	+ 14	7.93	8.56	−0.23	+0.05	70.32	70.12						
30	12/12 to 16/39	263	373	739	+103	420	188	667	+ 59	8.24	8.56	−0.54	+0.12	69.83	69.83	I	10.0	3.0	3.7		

Dietary intake of periods 1 to 30 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 53.5 grams, fat (estimated from tables) = 95.2 grams, carbohydrate (estimated from tables) = 241.6 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat, and 4 for 1 gram of carbohydrate) = 2,037. In addition sugar was given *ad lib*, with an average intake of 30 grams (120 calories).

* Initial weight 74.65 kgm.

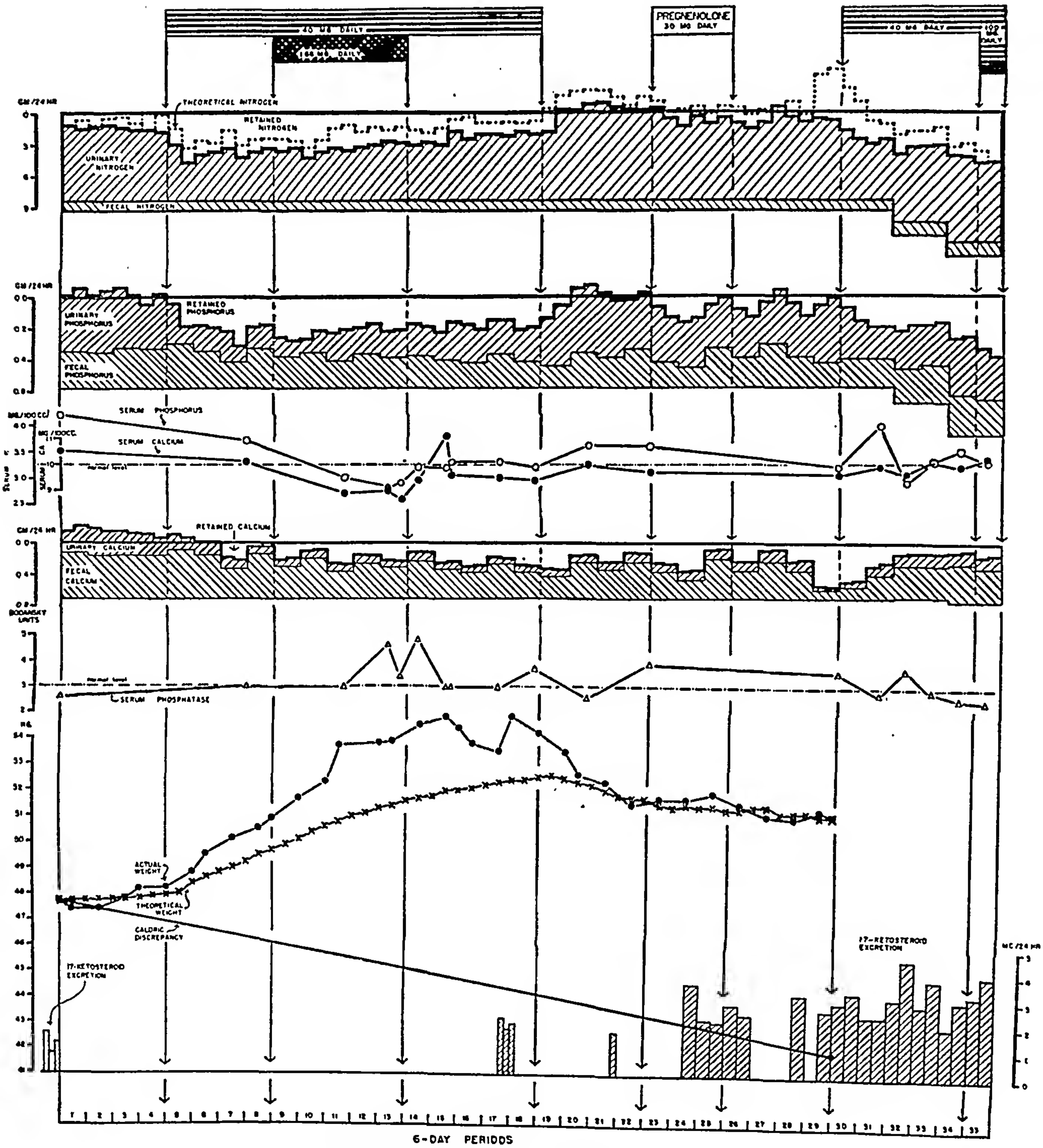

FIG. 4. CASE 4 (R. W., M.G.H. 319940): EFFECT OF METHYL TESTOSTERONE ALONE AND IN COMBINATION WITH ESTRADIOL BENZOATE, AND OF PREGNENOLONE ON NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES, ON SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS, ON BODY WEIGHT, AND ON URINARY 17-KETOSTEROID EXCRETION IN A FEMALE PATIENT WITH POST-MENOPAUSAL OSTEOPOROSIS

For discussion, see text.

nitrogen balance, which indicates that there is some constant error throughout. Part of the error may be in the fecal nitrogen excretion which was not analyzed, but taken as 10 per cent of the nitrogen intake. In the absence of analyzed values, it would have been preferable, and the discrepancy would have been cut down, had we used the value of 1.283 grams per 24 hours, the average fecal nitrogen value for adults regardless of intake (9). The major part of the discrepancy is probably to be attributed to errors in the intakes. The daily diet was

TABLE IV

Data for case 4 (R.W., M.G.H. 319940)

Period number	Date	Calcium				Phosphorus				Nitrogen				Body weight		Urinary 17-ketosteroids	Serum				Treatment		
		Urinary	Fecal	Intake	Balance	Urinary	Fecal	Intake	Balance	Urinary	Intake	Balance	Theoretical balance	Measured	Theoretical		Day of Period	Calcium	Phosphorus	Alkaline Phosphates	Methyl testosterone (p.o.)	Estradiol benzoate (i.m.)	Pregnenolone (i.m.)
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*				*kgm.*		*mgm. per 24 hr.*		*mgm. per 100 ml.*		*B.U.*			
	9/17/41																	10.6	3.1	3.7			
	10/15 to 16/41															1.6							
	10/16 to 17/41															0.8							
	10/17 to 18/41													47.70		1.2							
1	10/18 to 20/41	274	584	708	−144	347	240	584	− 3	7.15	9.31	+1.23	+1.0	47.37	47.70		III	10.5	4.2	2.4	None	None	None
	10/21 to 23/41	339	584	708	−212	405	240	584	− 61	6.86	9.31	+1.52	+0.6		47.73								
2	10/24 to 26/41	343	547	708	−182	358	234	584	− 8	6.99	9.31	+1.39	+1.2	47.40	47.76								
	10/27 to 29/41	316	547	708	−155	398	234	584	− 48	7.03	9.31	+1.35	+0.4		47.77								
3	10/30 to 11/1/41	315	554	708	−161	385	258	584	− 59	7.26	9.31	+1.12	+0.3	47.83	47.80								
	11/ 2 to 4/41	284	554	708	−130	327	258	584	− 1	6.78	9.31	+1.60	+0.9	48.16	47.84								
4	11/ 5 to 7/41	279	552	708	−123	289	254	584	+ 41	6.80	9.31	+1.58	+1.5		47.88								
	11/ 8 to 10/41	215	552	708	− 59	347	254	584	− 17	6.67	9.31	+1.71	+0.18	48.26	47.93								
5	11/11 to 13/41	207	623	708	−122	254	288	584	+ 42	5.39	9.31	+2.99	+1.5		48.09						40 mgm. daily		
	11/14 to 16/41	162	623	708	− 77	144	288	584	+185	3.77	9.31	+4.61	+3.3	48.79	48.39								
6	11/17 to 19/41	156	563	708	− 11	169	240	584	+175	4.49	9.31	+3.89	+2.7	49.55	48.63								
	11/20 to 22/41	150	563	708	− 5	153	240	584	+191	5.00	9.31	+3.38	+2.9		48.83								
7	11/23 to 25/41	142	380	708	+186	198	172	584	+214	5.30	9.31	+3.08	+1.7	50.14	49.00								
	11/26 to 28/41	112	380	708	+216	100	172	584	+312	4.16	9.31	+4.22	+3.1		49.27								
8	11/29 to 12/1/41	99	583	708	+ 26	140	257	584	+187	4.77	9.31	+3.61	+2.6	50.53	49.48								
	12/ 2 to 4/41	94	583	708	+ 31	150	257	584	+177	5.14	9.31	+3.24	+2.4	50.86	49.67								
9	12/ 5 to 7/41	92	420	708	+196	121	204	584	+262	4.50	9.31	+3.87	+2.5		49.94							1.66 mgm. daily	
	12/ 8 to 10/41	100	420	708	+188	108	201	584	+275	4.82	9.31	+3.56	+2.7	51.70	50.12								
10	12/11 to 13/41	96	528	708	+ 84	90	230	584	+264	4.24	9.31	+4.14	+3.3		50.38								
	12/14 to 16/41	102	528	708	+ 78	138	230	584	+216	4.76	9.31	+3.62	+2.6	52.34	50.59								
11	12/17 to 19/41	103	356	708	+249	183	174	584	+227	5.12	9.31	+3.26	+1.5	53.73	50.78								
	12/20 to 22/41	97	356	708	+255	204	174	584	+206	5.00	9.31	+3.28	+1.2		50.98								
12	12/23 to 25/41	105	465	708	+138	173	222	584	+189	5.30	9.31	+3.10	+1.8		51.15								
	12/26 to 28/41	100	465	708	+143	195	222	584	+167	5.44	9.31	+2.94	+1.4	53.83	51.31								
13	12/29 to 31/41	92	402	708	+214	168	195	584	+224	5.83	9.31	+2.55	+1.7	53.94	51.43		III	8.9	3.0	2.9			
	1/ 1 to 3/42	85	402	708	+221	187	195	584	+202	5.67	9.31	+2.71	+1.4		51.57		II	9.0	2.8	4.5			
14	1/ 4 to 6/42	111	473	708	+124	204	209	584	+174	5.47	9.31	+2.91	+1.7	54.48	51.72		II	8.7	2.9	3.3		None	
	1/ 7 to 9/42	111	473	708	+124	188	209	584	+187	5.71	9.31	+2.67	+1.9		51.85		III	9.4	3.2	4.7			
15	1/10 to 12/32	88	388	708	+232	179	182	584	+223	5.33	9.31	+2.85	+1.5	54.82	52.00								
	1/13 to 15/42	94	388	708	+226	246	182	584	+156	6.81	9.31	+1.57	+0.64	54.44	52.04								
16	1/16 to 18/42	78	342	708	+288	246	164	584	+174	6.04	9.31	+2.34	+0.45		52.14		I	9.6	3.3	2.9			
	1/19 to 21/42	90	342	708	+276	218	164	584	+202	6.40	9.31	+1.98	+0.96	53.86	52.22								
17	1/22 to 24/42	85	454	708	+172	214	219	584	+151	6.21	9.31	+2.17	+0.97		52.29								
	1/25 to 27/42	78	451	708	+179	213	219	584	+152	6.37	9.31	+2.01	+0.94	53.52	52.37	2.2 1.8 2.0	III	9.5	3.3	2.9			
18	1/28 to 30/42	95	342	708	+271	240	174	584	+203	6.57	9.31	+1.81	+1.0	54.83	52.42								
	1/31 to 2/2/42	86	342	708	+280	220	171	584	+193	6.33	9.31	+2.05	+0.8		52.50								

TABLE IV—*Continued*

Period number	Date	Calcium: Urinary	Calcium: Fecal	Calcium: Intake	Calcium: Balance	Phosphorus: Urinary	Phosphorus: Fecal	Phosphorus: Intake	Phosphorus: Balance	Nitrogen: Urinary	Nitrogen: Intake	Nitrogen: Balance	Nitrogen: Theoretical balance	Body weight: Measured	Body weight: Theoretical	Urinary 17-ketosteroids	Serum: Day of Period	Serum: Calcium	Serum: Phosphorus	Serum: Alkaline Phosphates	Treatment: Methyl testosterone (p.o.)	Treatment: Estradiol benzoate (i.m.)	Treatment: Pregnenolone (i.m.)
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*				*kgm.*		*mgm. per 24 hr.*		*mgm. per 100 ml.*		*B.U.*			
19	2/ 3 to 5/42	98	298	708	+312	308	141	584	+135	6.59	9.31	+1.79	−0.31	54.20	52.56		II	9.4	3.2	3.6	None	None	None
	2/ 6 to 8/42	84	298	708	+326	391	141	584	+52	8.39	9.31	−0.01	−1.6		52.46								
20	2/ 9 to 11/42	82	466	708	+160	412	224	584	−52	8.40	9.31	−0.02	−1.9	53.47	52.35								
	2/12 to 14/42	97	466	708	+145	428	224	584	−68	9.19	9.31	−0.81	−2.1	52.61	52.18								
21	2/15 to 17/42	92	379	708	+237	409	195	584	−20	9.35	9.31	−0.97	−2.0		52.00		II	10.0	3.6	2.5			
	2/18 to 20/42	94	379	708	+235	369	195	584	+20	8.87	9.31	−0.49	−1.4	52.31	51.86	1.7							
22	2/21 to 23/42	106	470	708	+132	324	247	584	+13	8.63	9.31	−0.25	−0.8		51.73								
	2/24 to 26/42	102	470	708	+136	366	247	584	−29	8.51	9.31	−0.13	−1.4	51.52	51.62								
23	2/27 to 3/1/42	101	350	708	+257	362	166	584	+56	8.81	9.31	−0.43	−1.1	51.69	51.48		I	9.8	3.6	3.8			30 mgm. daily
	3/ 2 to 4/42	100	350	708	+258	289	166	584	+129	7.77	9.31	+0.60	0		51.43								
24	3/ 5 to 7/42	110	238	708	+360	287	126	584	+171	7.03	9.31	+1.35	−0.13	50.60	51.45								
	3/ 8 to 10/42	123	238	708	+347	318	126	584	+140	7.92	9.31	+0.46	−0.49	51.59	51.39	3.6							
25	3/11 to 13/42	117	506	708	+85	280	251	584	+53	7.25	9.31	+1.13	+0.61	51.99	51.39	2.2							
	3/14 to 16/42	129	506	708	+73	329	251	584	+4	7.82	9.31	+0.56	−0.48	51.35	51.34	2.1							
26	3/17 to 19/42	122	357	708	+229	311	189	584	+84	7.18	9.31	+1.20	−0.45	51.48	51.34	2.8							None
	3/20 to 22/42	127	357	708	+224	263	189	584	+132	6.65	9.31	+1.73	+0.3	51.37	51.39	2.4							
27	3/23 to 25/42	144	472	708	+92	272	274	584	+38	7.34	9.31	+1.04	−0.1	51.12	51.38								
	3/26 to 28/42	163	472	708	+73	346	274	584	−36	8.71	9.31	−0.33	0	51.15	51.26								
28	3/29 to 31/42	126	365	708	+217	348	193	584	+43	7.86	9.31	+0.52	−0.97	51.06	51.20								
	4/ 1 to 3/42	132	365	708	+211	266	193	584	+125	7.45	9.31	+0.93	+0.3	51.06	51.18	3.2							
29	4/ 4 to 6/42	58	141	708	+509	375	187	584	+22	7.83	9.31	+0.55	−3.5	51.35	51.13								
	4/ 7 to 9/42	50	141	708	+517	419	187	584	−22	7.64	9.31	+0.74	−4.0	51.10	51.10	2.6							
30	4/10 to 12/42	56	115	708	+537	320	151	584	+113	6.67	9.31	+1.70	−2.3	50.86		2.9	I	9.6	3.2	3.5	40 mgm. daily		
	4/13 to 15/42	71	115	708	+522	240	151	584	+193	5.69	9.31	+2.69	−1.0	51.10		3.3							
31	4/16 to 18/42	119	297	708	+292	205	180	584	+199	5.41	9.31	+2.97	+0.79	51.81		2.4							
	4/19 to 21/42	161	297	708	+250	204	180	584	+200	5.75	9.31	+2.63	+1.1	52.26		2.4	III	9.9	4.0	2.7			
32	4/22 to 24/42	150	412	700	+138	246	216	682	+220	6.53	11.80	+4.09	+2.2	52.31		3.1							
	4/25 to 27/42	163	412	700	+125	282	216	682	+184	7.16	11.80	+3.46	+1.8	52.27		4.6							
33	4/28 to 30/42	159	405	700	+136	255	238	682	+189	7.30	11.80	+3.32	+1.8	52.38		2.8	I	9.6	2.9	3.6			
	5/ 1 to 3/42	161	405	700	+134	273	238	682	+171	7.35	11.80	+3.27	+1.5	52.84		3.8							
34	5/ 4 to 6/42	166	478	745	+101	365	255	890	+270	8.29	13.9	+4.22	+3.3	52.67		2.0	I	10.1	3.3	2.8			
	5/ 7 to 9/42	157	478	745	+110	379	255	890	+256	8.42	13.9	+4.09	+3.0	52.19		3.0							
35	5/10 to 12/42	160	431	745	+154	329	232	890	+329	7.66	13.9	+4.85	+3.7	52.56		3.2	I	9.9	3.5	2.5	100 mgm. daily		
	5/13 to 15/42	170	431	745	+144	279	232	890	+379	7.71	13.9	+4.80	+4.6	52.95		4.0					100 mgm. daily		
	5/16/42																	10.2	3.3	2.1			

Dietary intake of periods 1 to 31 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 52.5 grams, fat (estimated from tables) = 63.5 grams, carbohydrate (estimated from tables) = 207.5 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat, and 4 for 1 gram of carbohydrate) = 1,611; periods 32 to 33: protein = 63.7 grams, fat = 51.3 gram, carbohydrate = 197.9 gram, calories = 1,508; periods 34 to 35; protein 86.9 grams, fat = 61.1 grams, carbohydrate = 178.7 grams, calories = 1,612. In addition throughout sugar was given *ad lib*, with an average intake of 30 grams (120 calories).

analyzed twice with the following results: analysis October 1941: calcium 71 mgm., phosphorus 584 mgm., and nitrogen 9.31 grams; analysis February 2, 1944: calcium 64 mgm., phosphorus 611 mgm., and nitrogen 8.40 grams. Figure 4 was constructed from the analysis of 1941; had it been constructed from the analysis of 1944, the discrepancy would have been almost eliminated. Thus, if one recalculates on the basis of the 1944 analysis the theoretical nitrogen balance of period 4b, and in addition uses the value of 1.283 grams for the fecal nitrogen instead of 10 per cent of the intake, one obtains the values +0.65 and +0.45 grams for the theoretical and actual nitrogen balances, respectively, in contrast to the values of +0.18 and +1.71 grams. Since the above discrepancy is fairly constant, it does not affect the trends induced by treatment.

Figure 4 is self-explanatory. To be noted are: (*1*) the decrease in the nitrogen, phosphorus, and calcium excretions with methyl testosterone therapy, and the rebound of nitrogen and phosphorus excretions on cessation of therapy; (*2*) the fact that the fecal, as well as the urinary, excretions of both calcium and phosphorus were reduced under methyl testosterone therapy; (*3*) the fact that there was not an immediate rebound of the calcium excretion following cessation of methyl testosterone therapy; (*4*) the further improvement in the calcium balance, but not in the nitrogen balance, when estradiol benzoate therapy was added to the methyl testosterone therapy (periods 9 to 13); (*5*) the fall in serum phosphorus level with methyl testosterone and especially with estradiol benzoate therapy; (*6*) the definite tendency of the serum calcium level to parallel the serum phosphorus level (see also Figure 2); and (*7*) the failure of the serum phosphatase level to show a significant change. The effect of the pregnenolone therapy is inconclusive; it did not significantly affect the very low 17-ketosteroid excretion. No explanation is forthcoming in periods 29 and 30 for the low fecal calcium excretions not associated with low nitrogen and phosphorus excretions; as a result, the data during periods 30 through 36 are difficult to interpret. The actual and theoretical weight curves suggest that there was retention of extracellular fluid with methyl testosterone therapy which was augmented when estradiol benzoate therapy was added. Pregnenolone therapy had a minimal effect on extracellular fluid retention.

Case 5. Post-Menopausal Osteoporosis; Artificial Menopause; Paget's Disease; Diethylstilbestrol and Progesterone Therapy.

The metabolic data of Case 5 are given in Figure 5 and Table V. The study, conducted in 6-day periods, consisted of: (*1*) three control periods; (*2*) five periods on 1 mgm. of diethylstilbestrol by mouth daily; (*3*) seven periods on 15 mgm. of diethylstilbestrol by mouth daily, with an increase in the diet in the last 3 of these; (*4*) six periods with the same dosage of diethylstilbestrol in which progesterone by injection was given in addition (25 mgm. daily for the first 4 of these periods, and 100 mgm. daily for the last 2); and (*5*) three periods on 15 mgm. of diethylstilbestrol daily alone.

This patient was selected for the study not only because she had marked osteoporosis from an artificial menopause 30 years before, but because she had, in addition, Paget's disease. The primary pathologic process of the Paget's disease, bone destruction, was not being responded to with the usual amount of increased bone formation because of the menopause (4). Therefore, it was thought that any action of estrogen to stimulate bone formation would be magnified in this patient.

Figure 5 is self-explanatory. To be noted are: (*1*) the markedly negative calcium and phosphorus balances during the control periods; (*2*) the marked improvement of these balances with 1 mgm. of diethylstilbestrol daily; (*3*) the further improvement with 15 mgm. of diethylstilbestrol daily; (*4*) the lack of effect of progesterone on the calcium and phosphorus balances; (*5*) the high serum phosphorus before treatment; (*6*) the tendency of the serum phosphorus to fall during treatment; (*7*) the failure of the serum phosphatase to rise with improvement of the calcium balance; (*8*) the tendency of the 17-ketosteroid excretion to rise with progesterone; (*9*) the failure of the "11-oxysteroid" excretion[4] to fluctuate outside of the normal range with therapy; (*10*) the striking fall[5] in the urinary follicle-stimulating hormone (FSH) excretion with diethylstilbestrol therapy; and (*11*) the subsequent rise in the FSH excretion when progesterone therapy was superimposed on the diethylstilbestrol therapy. The increase in the positive nitrogen balance and the increase in weight during periods 22 to 24 may be indications that progesterone was acting unfavorably on the nitrogen balance (12). Not explained is the rise in FSH excretion in periods 23 and 24.

B. Senile osteoporosis

Case 6. Senile Osteoporosis in a Male of 72; Testosterone Propionate and Estradiol Benzoate Therapy.

The metabolic data of Case 6, which comprise studies done on 290 of 530 consecutive days, are shown in Figure 6 and Table VI. The study, conducted in 5-day periods, consisted of: (*1*) five control periods; (*2*) five periods on testosterone propionate, 25 mgm. by injection daily; (*3*) five periods in which estradiol benzoate 1.66 mgm. by injection on alternate days was added to the testosterone propionate therapy; (*4*) five periods back on testosterone propionate alone; (*5*) seven control periods off all medication; (*6*) five periods on estradiol benzoate 1.66 mgm. by injection twice daily; (*7*) ten days without collections on the same medication; (*8*) two more periods on the same medication; (*9*) ninety-three days at home on estradiol benzoate 3.32 mgm. by injection 3 times

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[4] These observations were carried out by Dr. Nathan B. Talbot with his method (10). The normal range is 0.10 to 0.35 mgm. per 24 hours.

[5] The level fell from 200–300 units per day to less than 6 units per day. Normal range of FSH excretion is 6 to 50 mouse units per day (11).

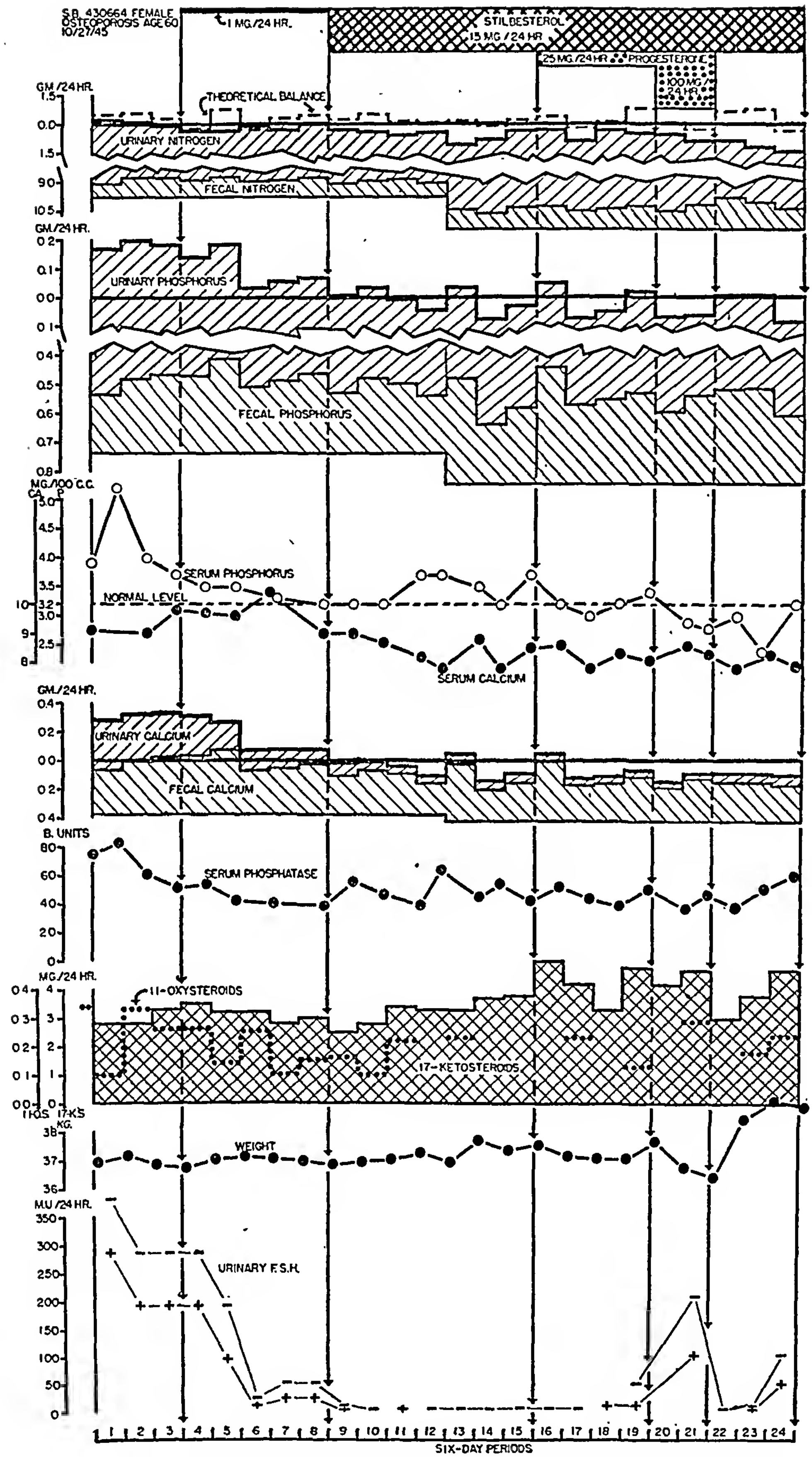

FIG. 5. CASE 5 (S. B., M.G.H. 430664): EFFECT OF DIETHYLSTILBESTROL ALONE AND IN COMBINATION WITH PROGESTERONE ON NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES, ON SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS, ON BODY WEIGHT, AND ON URINARY 17-KETOSTEROID, "11-OXYSTEROID," AND FOLLICLE-STIMULATING HORMONE EXCRETION IN A FEMALE PATIENT WITH POST-MENOPAUSAL OSTEOPOROSIS AND PAGET'S DISEASE

For discussion, see text.

TABLE V

Data for case 5 (S.B., M.G.H. 430664)

Period number	Date	Calcium				Phosphorus				Nitrogen					Body weight			Follicle stimulating hormone excretion in urine		Serum				Treatment	
		Urinary	Fecal	Intake	Balance	Urinary	Fecal	Intake	Balance	Urinary	Fecal	Intake	Balance	Theoretical balance	Measured	Urinary 17-ketosteroids	Urinary 11-oxysteroids	Positive	Negative	Day of period	Calcium	Phosphorus	Alkaline phosphatase	Diethylstilbestrol (p.o.)	Progesterone (i.m.)
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*					*kgm.*	*mgm. per 24 hr.*		*mouse units per 24 hr.*			*mgm. per 100 ml.*		*B.U.*		
	10/23/45																				8.8	3.7	61.5	None	None
	10/24/45															3.4	0.34								
	10/27/45														36.9										
1	10/27 to 11/1/45	341	315	377	−279	696	212	737	−171	9.29	0.74	9.83	−0.20	−0.44	37.1	2.8	0.10	+288	−381	I	9.1	3.9	75.7		
2	11/ 2 to 7/45	324	373	377	−320	678	257	737	−198	8.91	1.03	9.83	−0.11	−0.56	36.9	2.8	0.33	+192	−288	I		5.2	82.8		
3	11/ 8 to 13/45	296	406	377	−325	646	275	737	−184	8.72	1.03	9.83	+0.08	−0.32	36.7	3.3	0.26	+192	−288	I	9.0	4.0	61.2		
4	11/14 to 19/45	277	413	377	−313	607	271	737	−141	8.53	0.94	9.83	+0.36	+0.22	37.0	3.5	0.26	+192	−288	I	9.8	3.7	51.5	1 mgm. daily	
5	11/20 to 25/45	188	454	377	−265	592	329	737	−184	8.44	1.03	9.83	+0.36	−0.76	37.1	3.2	0.14	+ 96	−192	I	9.7	3.5	53.7		
6	11/26 to 12/1/45	140	306	377	− 69	539	230	737	− 32	8.94	0.80	9.83	+0.09	+0.03	37.1	3.2	0.25	+ 13	− 26	I	9.6	3.5	12.1		
7	12/ 2 to 7/45	127	324	377	− 74	542	251	737	− 56	8.70	0.86	9.83	+0.27	−0.28	37.0	2.8	0.10	+ 26	− 52	II	10.4	3.3	39.9		
8	12/ 8 to 13/45	101	351	377	− 75	527	277	737	− 67	8.84	1.01	9.83	−0.02	−0.44	36.8	3.0	0.15	+ 26	− 52						
9	12/14 to 19/45	88	275	377	+ 14	535	209	737	− 7	8.74	0.77	9.83	+0.31	−0.21	36.9	2.5	0.16	+ 6.5	− 13	I	9.0	3.2	39.4	15 mgm. daily	
10	12/20 to 25/45	64	308	377	+ 5	510	261	737	− 34	8.53	0.88	9.83	+0.42	−0.53	37.0	2.8	0.10		− 6.5	I	9.0	3.2	55.6		
11	12/26 to 31/45	54	287	377	+ 36	486	243	737	+ 8	8.31	0.95	9.83	+0.57	−0.15	37.3	3.4	0.22	+ 6.5		I	8.7	3.2	46.8		
12	1/ 1 to 6/46	54	221	377	+102	496	196	737	+ 45	8.63	0.75	9.83	+0.45	−0.09	36.9	3.3			− 6.5	II	8.2	3.7	39.7		
13	1/ 7 to 12/46	68	393	417	− 44	513	361	842	− 32	9.34	1.02	11.46	+1.10	−0.15	37.7	3.3	0.23		− 6.5	I	7.8	3.7	63.7		
14	1/13 to 18/46	59	218	417	+140	562	202	842	+ 78	9.78	0.84	11.46	+0.84	+0.12	37.3	3.7			− 6.5	II	8.8	3.5	41.9		
15	1/19 to 24/46	62	269	417	+ 86	552	260	842	+ 30	9.50	1.14	11.46	+0.37	−0.19	37.5	3.8			− 6.5	I	7.8	3.2	54.0		
16	1/25 to 30/46	52	415	417	− 50	492	398	842	− 48	9.95	1.19	11.46	+0.32	−0.34	37.1	5.0			− 6.5	I	8.5	3.7	41.9		25 mgm. daily
17	1/31 to 2/5/46	41	254	417	+122	495	273	842	+ 74	9.59	0.97	11.46	+0.90	+0.19	37.1	4.2	0.23		− 6.5	I	8.6	3.2	52.1		25 mgm. daily
18	2/ 6 to 11/46	42	264	417	+111	506	288	842	+ 48	10.07	1.06	11.46	+0.33	−0.10	37.1	3.3		+ 13		I	7.8	3.0	41.0		25 mgm. daily
19	2/12 to 17/46	48	300	417	+ 69	546	314	842	− 18	9.86	1.11	11.46	+0.48	−0.76	37.7	4.8	0.13	+ 13	− 52	I	8.3	3.2	38.8		25 mgm. daily
20	2/18 to 23/46	41	229	417	+147	526	246	842	+ 70	9.97	0.97	11.46	+0.53	−0.01	36.7	4.2				I	8.1	3.4	50.2		100 mgm. daily
21	2/24 to 3/1/46	38	289	417	+ 90	479	299	842	+ 64	9.28	1.25	11.46	+0.93	+0.28	36.4	4.7	0.29	+101	−208	II	8.6	2.9	37.7		100 mgm. daily
22	3/ 2 to 7/46	62	269	417	+ 86	526	323	842	− 7	8.93	1.63	11.46	+0.89	−0.73	38.5	3.0			− 6.5	I	8.3	2.8	47.0		None
23	3/ 8 to 13/46	57	272	417	+ 88	524	325	842	− 7	9.01	1.23	11.46	+1.22	−0.75	39.1	3.8	0.18	+ 6.5	− 13	I	7.8	3.0	38.0		
24	3/14 to 19/46	67	248	417	+102	523	233	842	+ 86	8.99	1.04	11.46	+1.43	+0.51	38.9	4.7	0.24	+ 52	−101	I	8.3	2.4	51.2		
	3/20/46																				7.9	3.2	60.0		

Dietary intake of periods 1 to 12 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 61.4 grams, fat (estimated from tables) = 67.1 grams, carbohydrate (estimated from tables) = 125.1 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat, and 4 for 1 gram of carbohydrate) = 1,350; periods 13 to 24: protein = 71.2 grams, fat = 69.4 grams, carbohydrate = 173.4 grams, calories = 1,603.

TABLE VI

Data for case 6 (M.H., M.G.H. 278511)

Period number	Date	Calcium				Phosphorus				Nitrogen				Body weight		Urinary 17-ketosteroids	Serum				Treatment	
		Urinary	Fecal	Intake	Balance	Urinary	Fecal	Intake	Balance	Urinary	Intake	Balance	Theoretical balance	Measured	Theoretical		Day of period	Calcium	Phosphorus	Alkaline phosphatase	Estradiol benzoate (i.m.)	Testosterone propionate (i.m.)
		mgm. per 24 hr.				mgm. per 24 hr.				grams per 24 hr.				kgm.		mgm. per 24 hr.		mgm. per 100 ml.		B.U.		
	12/14/40																	10.3	4.4	4.2	None	None
	12/18/40																	10.7	2.5	4.2		
	12/27/40																	10.2	2.9	5.0		
	12/31/40													70.34		7.2						
1	1/ 1 to 5/41	165	338	701	+198	310	219	611	+ 82	6.92	8.4	+0.64	−0.25	70.24	70.02							
2	1/ 6 to 10/41	189	417	701	+ 95	326	190	611	+ 95	6.96	8.4	+0.60	0	70.37	69.84							
3	1/11 to 15/41	150	397	701	+154	310	197	611	+104	6.44	8.4	+1.12	+0.41	70.39	69.62							
4	1/16 to 20/41	164	421	701	+116	244	242	611	+125	6.37	8.4	+1.19	+1.01	70.22	69.49	IV* 7.0						
5	1/21 to 25/41	141	397	701	+163	276	196	611	+139	5.81	8.4	+1.75	+0.87	69.85	69.34							
6	1/26 to 30/41	152	288	701	+261	207	153	611	+251	5.51	8.4	+2.05	+1.80	70.44	69.32							25 mgm. daily
7	1/31 to 2/4/41	137	317	701	+247	132	162	611	+317	4.43	8.4	+3.13	+2.91	70.99	69.47							
8	2/ 5 to 9/41	117	173	701	+411	126	94	611	+391	4.05	8.4	+3.51	+2.79	70.98	69.59							
9	2/10 to 14/41	99	374	701	+228	115	192	611	+304	3.49	8.4	+4.07	+2.85	71.35	69.73							
10	2/15 to 19/41	129	280	701	+292	130	153	611	+328	4.36	8.4	+3.20	+2.73	71.08	69.84							
11	2/20 to 24/41	93	188	701	+420	124	105	611	+382	3.89	8.4	+3.67	+2.58	71.53	69.94	V 11.8	V	9.8	2.0	2.4	1.66 mgm. every other day	
12	2/25 to 3/1/41	107	190	701	+404	129	105	611	+377	4.60	8.4	+2.96	+2.63	71.74	70.04							
13	3/ 2 to 6/41	81	161	701	+459	161	84	611	+366	4.20	8.4	+3.36	+2.05	71.72	70.06							
14	3/ 7 to 11/41	96	192	701	+413	134	105	611	+372	4.26	8.4	+3.30	+2.47	71.87	70.14							
15	3/12 to 16/41	84	199	701	+418	170	121	611	+320	4.78	8.4	+2.78	+1.67	71.88	70.10							
16	3/17 to 21/41	105	160	701	+436	159	99	611	+353	4.45	8.4	+3.11	+2.03	71.39	70.12						None	
17	3/22 to 26/41	79	204	701	+418	184	120	611	+307	4.67	8.4	+2.89	+1.47	71.55	70.05							
18	3/27 to 31/41	97	230	701	+374	160	125	611	+326	4.94	8.4	+2.62	+2.09	71.27	70.08							
19	4/ 1 to 5/41	77	269	701	+355	163	161	611	+287	4.63	8.4	+2.93	+1.65	71.44	70.04		IV	10.5	1.8	2.5		
20	4/ 6 to 10/41	118	233	701	+350	186	152	611	+273	5.61	8.4	+1.95	+1.47	71.46	69.97	V 11.2	V	9.5	2.1	2.5		
21	4/11 to 15/41	81	207	701	+413	215	130	611	+266	5.08	8.4	+2.48	+0.90	70.21	69.82							None
22	4/16 to 20/41	95	260	701	+346	385	183	611	+ 43	7.84	8.4	−0.28	−1.95	69.43	69.26							
23	4/21 to 25/41	108	302	701	+291	448	163	611	0	9.13	8.4	−1.57	−2.17	68.51	68.66	II 4.8	II	10.2	3.1	3.1		
24	4/26 to 30/41	126	237	701	+338	367	135	611	+109	9.01	8.4	−1.45	−0.90	68.22	68.25							
25	5/ 1 to 5/41	147	352	701	+202	318	186	611	+107	8.23	8.4	−0.67	+0.09	67.97	67.98							
26	5/ 6 to 10/41	175	284	701	+242	330	180	611	+101	8.13	8.4	−0.57	−0.30	67.35	67.66	IV 7.2	IV	10.2	3.2	4.4		
27	5/11 to 15/41	191	245	701	+265	335	156	611	+120	7.93	8.4	−0.37	−0.18	67.37	67.37							
28	5/16 to 20/41	194	292	701	+215	303	161	611	+147	8.36	8.4	−0.80	+0.60	67.52			IV	10.4	2.8	3.1	3.32 mgm. daily	
29	5/21 to 25/41	171	209	701	+321	223	136	611	+252	7.29	8.4	+0.27	+1.38	67.62								
30	5/26 to 30/41	161	153	701	+387	257	88	611	+266	7.47	8.4	+0.09	+1.09	67.56								
31	5/31 to 6/4/41	132	282	701	+287	277	166	611	+168	7.42	8.4	+0.14	+0.37	67.27		III 3.8	III	10.2	2.8	3.8		
32	6/ 5 to 8/41	167	347	701	+187	309	216	611	+ 86	8.28	8.4	−0.72	−0.11	66.96								
	No collections for 10 days 6/16/41													69.14		2.4		9.6	2.2	2.5		
33	6/19 to 23/41	128	357	701	+216	369	201	611	+ 41	8.76	8.4	−1.20	−1.01	67.20								
34	6/24 to 27/41	139	199	701	+363	364	110	611	+137	8.09	8.4	−0.53	−0.66	66.75								

* Day of period.

TABLE VI—*Continued*

Period number	Date	Calcium Urinary	Calcium Fecal	Calcium Intake	Calcium Balance	Phosphorus Urinary	Phosphorus Fecal	Phosphorus Intake	Phosphorus Balance	Nitrogen Urinary	Nitrogen Intake	Nitrogen Balance	Nitrogen Theoretical balance	Body weight Measured	Body weight Theoretical	Urinary 17-ketosteroids	Serum Day of period	Serum Calcium	Serum Phosphorus	Serum Alkaline phosphatase	Treatment Estradiol benzoate (i.m.)	Treatment Testosterone propionate (i.m.)
		mgm. per 24 hr.				mgm. per 24 hr.				grams per 24 hr.				kgm.		mgm. per 24 hr.		mgm. per 100 ml.		B.U.		
	No collections for 93 days 9/26/41													69.12		2.8		10.1	2.7	3.0	3.32 mgm. three times a week	None
35	9/28 to 10/2/41	171	212	701	+318	347	129	611	+135	8.11	8.4	−0.55	−0.36	69.13								
36	10/ 3 to 7/41	169	394	701	+138	360	226	611	+ 25	7.70	8.4	−0.14	−0.66	69.20								
37	10/ 8 to 12/41	176	329	701	+196	339	184	611	+ 88	7.75	8.4	−0.19	−0.15	68.97								
38	10/13 to 17/41	233	444	701	+ 24	323	249	611	+ 39	7.35	8.4	+0.21	−0.41	68.61								
39	10/18 to 22/41	187	386	701	+128	301	211	611	+ 99	7.59	8.4	−0.03	+0.53	68.87								
40	10/23 to 27/41	142	445	701	+114	234	244	611	+133	6.82	8.4	+0.74	+1.14	69.15								25 mgm. daily
41	10/28 to 11/1/41	192	420	701	+ 89	180	200	611	+231	5.94	8.4	+1.62	+2.81	70.22			II	9.9	2.2	2.2		
42	11/ 2 to 6/41	200	312	701	+189	145	153	611	+313	4.95	8.4	+2.61	+3.27	70.19								
43	11/ 7 to 11/41	182	331	701	+188	188	171	611	+249	5.14	8.4	+2.42	+2.33	70.23								
44	11/12 to 16/41	216	194	701	+291	197	124	611	+290	5.57	8.4	+1.99	+2.17	70.18								
45	11/17 to 21/41	185	317	701	+199	226	175	611	+210	5.51	8.4	+2.05	+1.67	70.26								
46	11/22 to 26/41	208	268	701	+225	326	157	864	+381	10.06	17.37	+5.57	+1.01	70.60			V	9.3	1.9	2.7		
47	11/27 to 12/1/41	240	219	701	+242	345	159	864	+360	10.74	17.37	+4.89	+3.59	71.13								
48	12/ 2 to 6/41	235	331	701	+135	414	202	864	+248	11.37	17.37	+4.26	+2.71	71.15								
49	12/ 7 to 11/41	230	331	701	+140	509	221	864	+134	13.96	17.37	+1.67	+0.96	70.14			III	9.5	2.0	3.0		None
50	12/12 to 16/41	166	410	701	+125	558	286	864	+ 20	14.39	17.37	+1.24	−0.65	70.35								
51	12/17 to 21/41	179	491	701	+ 31	655	290	864	− 81	16.08	17.37	−0.45	−1.44	69.92								
	12/22/41 No collections for 91 days 3/21/42													69.86				9.3	2.8	2.6		
52	3/23 to 27/42	166	484	701	+ 51	335	295	611	− 19	8.13	8.4	−0.57	−0.66	69.66		III 5.2	I	10.3	2.3	2.6		
53	3/28 to 4/1/42	148	395	701	+158	332	263	611	+ 16	8.46	8.4	−0.90	−0.95	69.54								
54	4/ 2 to 6/42	83	141	701	+477	389	170	611	+ 52	8.47	8.4	−0.91	−2.80	69.16								
	No collections for 43 days 5/18/42													69.40		7.2	.	10.6	2.6	3.5	None	
55	5/20 to 24/42	182	476	701	+ 43	379	221	611	+ 11	7.68	8.4	−0.12	−0.15	68.98		I 7.4	I	10.2	3.1	2.7		
56	5/25 to 29/42	207	341	701	+153	411	194	611	+ 6	7.47	8.4	+0.09	−1.07	69.18			I	10.0	2.8	2.6		
57	5/30 to 6/3/42	240	301	701	+160	359	151	611	+101	7.09	8.4	+0.47	+0.31	68.86			I	10.0	3.0	3.5		
58	6/ 1 to 8/42	253	404	701	+ 44	339	227	611	+ 45	7.16	8.4	+0.40	+0.35	68.44			I	10.3	3.3	3.7		
	6/ 9/42															.		10.3	2.9	2.7		
	6/15/42															.		10.0	2.8	3.3		

Dietary intake of periods 1 to 45 and 52 to 58 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 52.5 grams, fat (estimated from tables) = 63.5 grams, carbohydrate (estimated from tables) = 207.5 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat, and 4 for 1 gram of carbohydrate) = 1,611; periods 46 to 51: protein = 108.6 grams, fat = 73.8 grams, carbohydrate = 221.0 gm., calories = 1,983. In addition throughout sugar was given *ad lib*, with an average intake of 30 grams (120 calories).

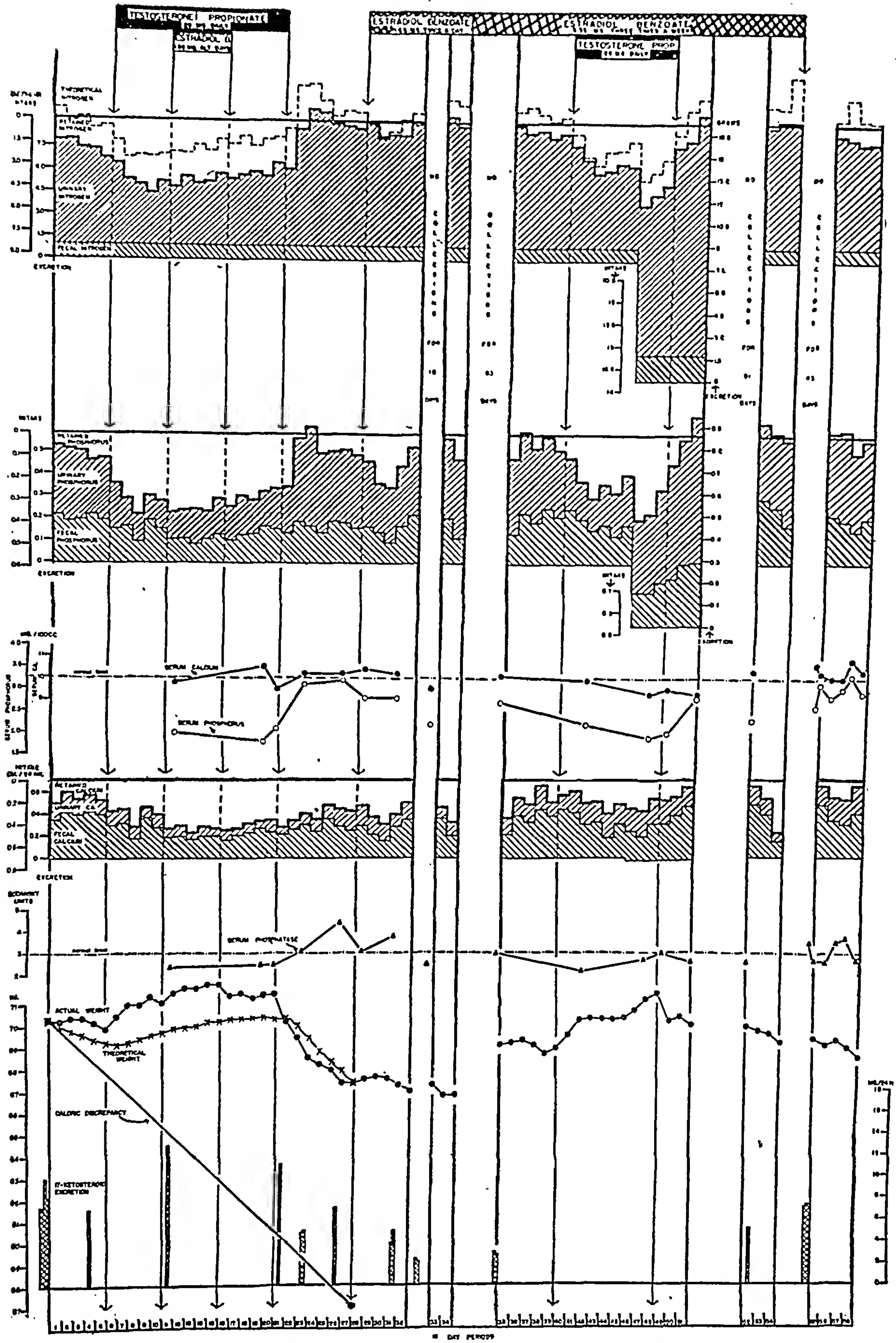

FIG. 6. CASE 6 (M. H., M.G.H. 278511): EFFECT OF TESTOSTERONE PROPIONATE ALONE AND IN COMBINATION WITH ESTRADIOL BENZOATE AND VICE VERSA ON NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES, ON SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS, ON BODY WEIGHT, AND ON URINARY 17-KETOSTEROID EXCRETION IN A MALE PATIENT WITH SENILE OSTEOPOROSIS

For discussion, see text.

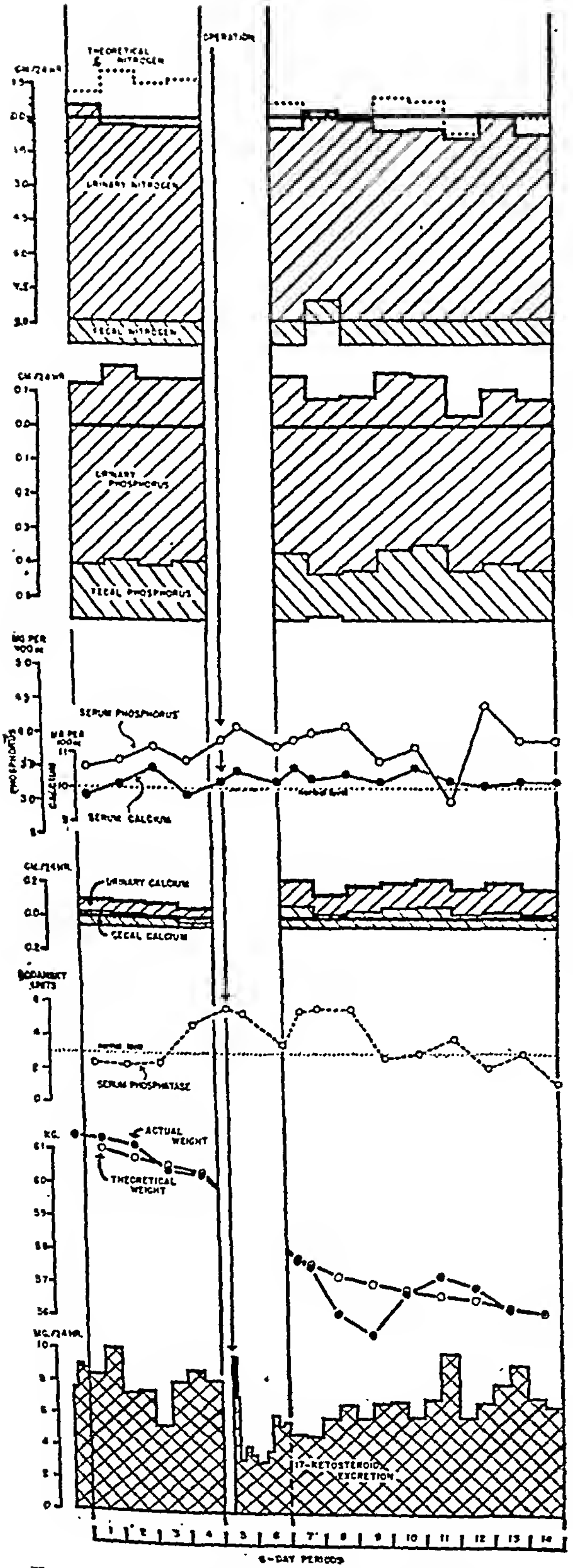

FIG. 7. CASE 7 (E. S., M.G.H. 360207): NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES; SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS; WEIGHT; AND URINARY 17-KETOSTEROID EXCRETION IN A FEMALE PATIENT WITH OSTEOPOROTIC PROCESS INDUCED BY OPERATION AND IMMOBILIZATION

For discussion, see text.

a week; (*10*) five periods on the same therapy; (*11*) nine periods in which testosterone propionate 25 mgm. intramuscularly daily was added to the estradiol benzoate therapy, during the last 3 of which periods the intakes of nitrogen and phosphorus were markedly increased; (*12*) three periods on the same diet and the same estradiol benzoate therapy but off testosterone propionate therapy; (*13*) ninety-one days at home on the same estradiol benzoate therapy; (*14*) three periods on the original diet without change in the estradiol therapy; (*15*) forty-three days at home off all medication; and finally (*16*) four control periods on the original diet without medication.

Figure 6 is self-explanatory. The observations as a whole confirm those noted in Cases 1 to 4 with post-menopausal osteoporosis.

Again, as in Case 4, the theoretical nitrogen balance as charted is consistently less positive than the actual nitrogen balance which suggests some constant error. This discrepancy is probably to be attributed to errors in the intakes and to estimation of the fecal nitrogen as 10 per cent of the nitrogen intake (see discussion under Case 4). Case 6 received the same diet as Case 4; this diet was analyzed twice with the results given in the discussion under Case 4. Figure 6 was constructed from the analysis of 1941; had it been constructed from the analysis of 1944, as is Table V, the discrepancy would have been almost eliminated. Thus, if one recalculates on the basis of the 1944 analysis, the theoretical nitrogen balance of period 5, and in addition uses the value of 1.283 grams for the fecal nitrogen instead of 10 per cent of the intake, one obtains the values + 0.87 and + 1.30 grams for the theoretical and actual nitrogen balances, respectively, in contrast to the values of + 0.41 and + 2.21 grams. As was pointed out in connection with Case 4, since the above discrepancy is fairly constant, it does not affect the trends induced by treatment.

To be noted especially in Figure 6 are: (*1*) the marked reduction in nitrogen, phosphorus, and calcium excretions with testosterone therapy; (*2*) the lack of rebound in the calcium excretion as opposed to nitrogen and phosphorus following cessation of testosterone therapy; (*3*) the further reduction in the phosphorus and especially in the calcium excretion, but not in the nitrogen excretion, when estradiol benzoate therapy was added to testosterone propionate therapy (periods 16 to 20); (*4*) the improvement in all 3 balances when testosterone propionate was added to estradiol benzoate therapy (periods 40 to 45); (*5*) reduction in the fecal as well as the urinary calcium and phosphorus excretions by both testosterone propionate and estradiol benzoate therapy; (*6*) the effect of both testosterone propionate and estradiol benzoate therapy in lowering the serum phosphorus level; (*7*) the failure of marked increases in the nitrogen and phosphorus bal-

TABLE VII

Data for case 7 (E.S., M.G.H. 360207)

Period number	Date	Calcium: Urinary	Calcium: Fecal	Calcium: Intake	Calcium: Balance	Phosphorus: Urinary	Phosphorus: Fecal	Phosphorus: Intake	Phosphorus: Balance	Nitrogen: Urinary	Nitrogen: Intake	Nitrogen: Balance	Nitrogen: Theoretical balance	Body weight: Measured	Body weight: Theoretical	Urinary 17-ketosteroids	Day of period	Serum: Calcium	Serum: Phosphorus	Serum: Alkaline phosphatase	Treatment
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*				*kgm.*		*mgm. per 24 hr.*		*mgm. per 100 ml.*		*B.U.*	
	6/21 to 22/42															7.6					
	6/22 to 23/42															9.0					
	6/23 to 24/42													61.40		8.8					
1	6/24 to 26/42	71	86	58	− 99	528	161	566	−123	9.43	9.90	−0.52	−1.11	61.32	61.02	8.4	III	9.8	3.5	2.4	None
	6/27 to 29/42															10.0					
2	6/30 to 7/2/42	71	80	58	− 93	566	175	566	−175	8.63	9.90	+0.28	−2.02	61.14	60.78	7.3	III	10.1	3.6	2.3	
	7/ 3 to 5/42															7.4					
3	7/ 6 to 8/42	71	63	58	− 82	547	159	566	−140	8.51	9.90	+0.40	−1.49	60.40	60.56	5.3	III	10.6	3.8	2.4	
	7/ 9 to 11/42															8.0					
4	7/12 to 14/42	80	35	58	− 57	535	171	566	−140	8.51	9.90	+0.40	−1.67	60.28	60.34	8.7	III	9.8	3.6	4.7	
	7/15 to 17/42															8.1					
	7/20/42 1st 12 hours after operation	Operation														9.6		10.2	3.9	5.8	None
	7/20/42 2nd 12 hours after operation	No collections for 12 days														7.2					
	7/22 to 23/42															3.3					
	7/23 to 24/42															4.1					
	7/24 to 25/42															3.6		10.5	4.1	5.3	
	7/25 to 26/42															3.1					
	7/26 to 27/42															3.2					
	7/27 to 28/42															3.8					
	7/28 to 29/42															6.0					
	7/29 to 30/42															5.4					
	7/30 to 31/42													57.73	57.79	5.6					
5	7/31 to 8/2/42	145	134	58	−221	517	197	566	−148	8.37	9.90	+0.54	−0.57	57.55	57.60	4.9	I	10.2	3.8	3.5	None
	8/ 3 to 5/42															4.8	I	10.6	3.9	5.5	
6	8/ 6 to 8/42	144	83	56	−167	515	121	555	− 81	8.23	8.91	−0.21	+0.05	56.14	57.28	5.9	I	10.3	4.0	5.7	
	8/ 9 to 11/42															6.7					
7	8/12 to 14/42	142	101	58	−185	507	145	566	− 86	8.72	9.90	+0.19	+0.09	55.50	57.03	5.9	I	10.4	4.1	5.7	
	8/15 to 17/42															6.8					
8	8/18 to 20/42	140	123	58	−205	517	203	566	−154	8.19	9.90	+0.72	−0.78	56.78	56.87	6.9	I	10.2	3.6	2.9	
	8/21 to 23/42															6.0					
9	8/24 to 26/42	144	128	58	−214	493	218	566	−145	8.26	9.90	+0.65	−0.57	57.27	56.70	7.3	I	10.6	3.8	3.0	
	8/27 to 29/42															9.9					
10	8/30 to 9/1/42	149	85	58	−176	452	144	566	− 30	7.93	9.90	+0.98	+0.79	56.93	56.59	6.0	I	10.2	3.0	3.9	
	9/ 2 to 4/42															6.9					
11	9/ 5 to 7/42	169	95	58	−206	505	164	566	−103	8.82	9.90	+0.09	0	56.30	56.32	8.0	I	10.1	4.4	2.2	
	9/ 8 to 10/42															9.2					
12	9/11 to 13/42	148	81	58	−171	497	145	566	− 76	8.03	9.90	+0.88	+0.13	56.20	56.20	7.2	I	10.2	3.9	3.0	
	9/14 to 15/42															6.7	I	10.2	3.9	1.3	

Dietary intake of periods 1 to 14 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 61.9 grams, fat (estimated from tables) = 80.9 grams, carbo-hydrate (estimated from tables) = 179.4 grams, calories (calculated from the values 4 for

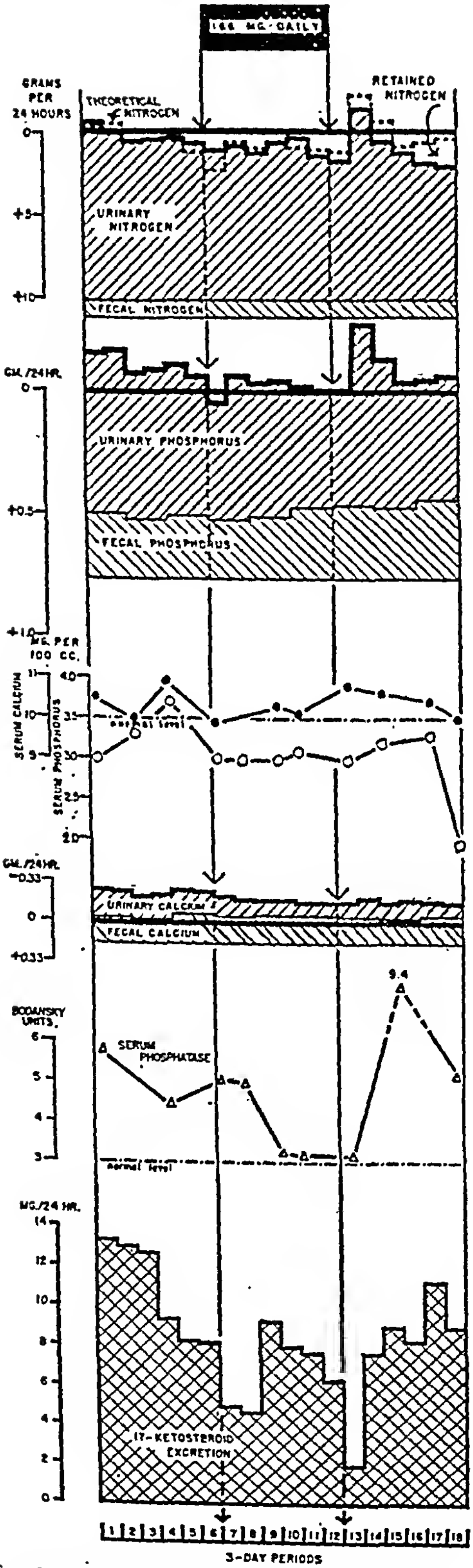

FIG. 8. CASE 8 (H. D., M.G.H. 382395): EFFECT OF ESTRADIOL BENZOATE ON NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES; ON SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE LEVELS; AND ON URINARY 17-KETOSTEROID EXCRETION IN A MALE PATIENT WITH OSTEOPOROTIC PROCESS INDUCED BY MULTIPLE FRACTURES, OPERATION, AND IMMOBILIZATION

For discussion, see text.

ances by increased diet to affect the calcium balance (periods 46, 47, 48); (*8*) the absence of any significant change in the serum phosphatase and calcium levels; (*9*) the fall in the urinary 17-ketosteroid level with estradiol benzoate therapy; and (*10*) the tendency to accumulate extracellular fluid during both testosterone propionate and estradiol benzoate therapy as suggested by the theoretical weight curves, with a prompt loss following the cessation of therapy.

C. *Osteoporosis resulting from disuse and/or adaptation syndrome*

Case 7. "Normal" Female; Effect of Orthopedic Operation; No Specific Therapy.

The metabolic data of Case 7 are shown in Figure 7 and Table VII. Throughout the entire experiment the patient was on a constant, neutral-ash, low calcium diet, except for the immediate post-operative period. She was up and active during the pre-operative period, and immobilized in a cast from the foot to the hip after operation. She underwent an arthrodesis of the right foot on the second day of period 5; there were no analyses for metabolic data during periods 5 and 6, but the 17-ketosteroid excretion was followed.

During the 4 control periods the patient was in negative calcium and phosphorus balance; the former was of the order of magnitude one would expect with patients on this diet (13). As expected, there was a marked increase in the calcium excretion after the operation, which persisted unabated to the end of the investigation (58 days after the operation) (14). The increase in calcium excretion was not entirely in the urine. The 17-ketosteroid excretion was normal pre-operatively, which confirms the contention that she was not debilitated; it rose immediately after operation, and then fell decidedly below the preoperative level for about 20 days. The pattern of response was thus similar to that encountered following any traumatizing event (15). The marked elevation in 17-ketosteroid excretion in period 11 coincided with the patient being allowed up in a wheel chair (16).

Periods 7 through 14 in this untreated case serve as a control for similar studies in Cases 8 and 9, who received estradiol therapy during the post-operative period (Figure 10).

Case 8. Multiple Traumatic Fractures with Operative Reduction of One in a Previously "Normal" Male; Effect of Estradiol Benzoate Therapy.

The metabolic data of Case 8 are shown in Figure 8 and Table VIII. The study, conducted in 3-day periods, consisted of; (*1*) six control periods; (*2*) six periods in which 1.66 mgm. of estradiol benzoate was given

TABLE VIII

Data for case 8 (H.D., M.G.H. 382395)
Fracture 11/15/42

Period number	Date	Calcium				Phosphorus				Nitrogen				Body weight	Urinary citrate	Urinary 17-ketosteroids	Serum				Treatment
		Urinary	Fecal	Intake	Balance	Urinary	Fecal	Intake	Balance	Urinary	Intake	Balance	Theoretical balance	Measured**			Day of period	Calcium	Phosphorus	Alkaline phosphatase	Estradiol benzoate (i.m.)
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*				*kgm.*	*mgm. per 24 hr.*			*mgm. per 100 ml.*		*B.U.*	
1	12/29 to 31/42	221	203	175	−249	647	266	760	−153	10.66	11.15	−0.62	−0.43		1151	13.3	I	10.5	3.0	3.4	None
2	1/ 1 to 3/43	225	203	175	−253	655	266	760	−161	9.97	11.15	+0.06	−0.53		1790	13.0					
2	1/ 4 to 6/43	200	196	175	−220	589	245	760	− 74	9.42	11.15	+0.61	+0.54		950	12.7	I	10.0	3.3	5.8	
4	1/ 7 to 9/43	208	196	175	−229	604	245	760	− 89	9.55	11.15	+0.48	+0.73		1162	9.4	III	10.9	3.7		
5	1/10 to 12/43	195	246	175	−266	605	260	760	−105	9.67	11.15	+0.37	+0.42		1190	8.3					
6	1/13 to 15/43	192	246	175	−263	550	260	760	− 50	9.41	11.15	+0.63	+1.21		1725	8.2					
	*																				
7	1/17 to 19/43	163	229	175	−217	474	244	760	+ 12	8.86	11.15	+1.17	+2.27		2001	5.0	I	9.9	3.0	4.5	1.66 mgm. daily
8	1/20 to 22/43	144	229	175	−197	577	244	760	− 61	9.17	11.15	+0.87	+0.57	60.02	1813	4.7	III		3.0	5.1	
9	1/23 to 25/43	137	221	175	−183	546	253	760	− 39	8.73	11.15	+1.31	+0.93		2280	9.3	III	10.3	3.0	5.1	
10	1/26 to 28/43	134	221	175	−180	547	253	760	− 40	9.36	11.15	+0.67	+0.75		2670	8.0					
11	1/29 to 31/43	114	223	175	−162	483	297	760	− 20	9.64	11.15	+0.39	+0.91		2500	7.7	I	10.1	3.1	3.3	
12	2/ 1 to 3/43	108	223	175	−156	471	297	760	− 8	8.59	11.15	+1.44	+1.05		2660	6.3					
13	2/ 4 to 6/43	121	225	175	−171	466	300	760	− 6	8.39	11.15	+1.65	+1.19		2388	2.0	I	10.8	3.0	3.2	None
14	2/ 7 to 9/43	182	225	175	−232	723	300	760	−263	11.36	11.15	−1.32	−2.21		1565	7.7					
15	2/10 to 12/43	129	212	175	−167	593	292	760	−135	9.49	11.15	+0.55	−0.63		1680	9.0	I	10.6	3.2	3.2	
16	2/13 to 15/43	142	212	175	−179	503	292	760	− 35	8.65	11.15	+1.39	+0.83	60.83	1880	8.3					
17	2/16 to 18/43	115	223	175	−163	477	324	760	− 41	8.55	11.15	+1.49	+0.60		1832	11.3	I	10.4	3.3	9.4	
18	2/19 to 21/43	112	223	175	−160	496	324	760	− 60	8.05	11.15	+1.98	+0.30	60.48	1880	9.0	III	10.0	1.9	5.2	

Dietary intake of periods 1 to 18 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 69.7 grams, fat (estimated from tables) = 50.1 grams, carbohydrate (estimated from tables) = 228.6 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat ,and 4 for 1 gram of carbohydrate) = 1,644.

* Collections on 1/16/43 omitted.

** Initial weight 11/15/42 between 70 and 75 kgm.

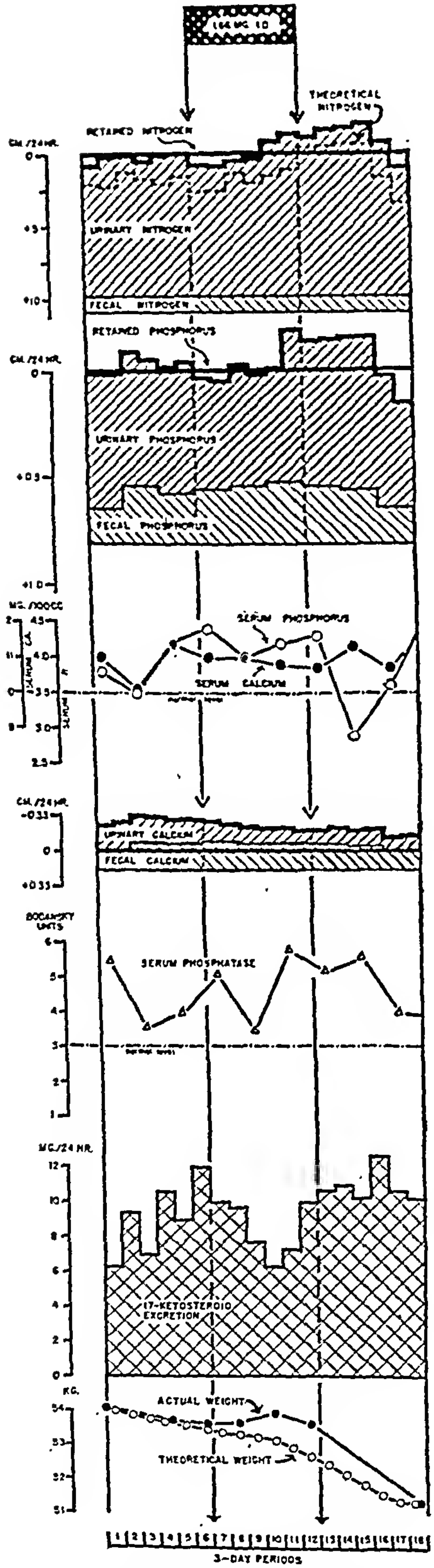

Fig. 9. Case 9 (C. M., M.G.H. 348774): Effect of Estradiol Benzoate on Nitrogen, Phosphorus, and Calcium Balances; on Serum Calcium, Phosphorus, and Alkaline Phosphatase Levels; on Urinary 17-Ketosteroid Excretion and on Weight in a Male Patient with Osteoporotic Process Induced by Operation and Immobilization

For discussion, see text.

daily by injection; and (*3*) six post-treatment control periods. The stool periods were analyzed 2 at a time.

Figure 8 is self-explanatory. The most important observations concern the calcium metabolism; these are better shown in Figure 10 and will be discussed below. Again, there was a fall in the serum phosphorus, and, if anything, a fall in the serum phosphatase. Of interest is the fall in 17-ketosteroids in period 13, followed by the rise in urinary nitrogen, phosphorus, and calcium in period 14; we believe these to be connected though unexplained phenomena.

Case 9. Bone Grafting Operation in an Ununited Femur of an Otherwise "Normal" Male; Effect of Estradiol Benzoate Therapy.

The metabolic data of Case 9 are shown in Figure 9 and Table IX. The study, conducted in 3-day periods, consisted of: (*1*) six control periods; (*2*) six periods in which 1.66 mgm. of estradiol benzoate was given daily by injection; and (*3*) six post-treatment control periods. The stool periods were analyzed 2 at a time.

Figure 9 is self-explanatory. The theoretical nitrogen balance shows a constant deviation from the measured nitrogen balance which suggests some constant error (*vide supra*). The calcium data, as in Case 8, are better shown in Figure 10, and will be discussed below. It should be noted that the serum phosphorus in this case, as opposed to all of the other cases, did not fall during estradiol therapy. The 17-ketosteroid excretion showed a tendency to fall during the estradiol benzoate therapy, which is also somewhat suggested in Figure 7.

Further analysis of calcium data of Cases 7, 8, and 9

In Figure 10 the calcium data of Cases 8 and 9 with estradiol benzoate therapy are compared with those of Case 7 without such therapy. It is quite clear that estradiol benzoate therapy resulted in a decrease in the urinary calcium excretion, but had little effect on the fecal calcium excretion during the 18 days of administration. However, the tendency for the fecal calcium to decrease in Case 9 after the therapy was stopped may well have been a delayed response to the therapy. The urinary citric acid values carried out and interpreted by Dr. Ephraim Shorr confirm his finding (17) of a rise during estrogen therapy.

D. Osteoporosis of Cushing's syndrome

Case 10. Cushing's Syndrome; Nephrolithiasis; Estradiol Benzoate and Testosterone Propionate Therapy.

The metabolic data of Case 10 are shown in graphic form in Figure 11. For data in tabular form for periods

TABLE IX

Data for case 9 (C.M., M.G.H. 348774)
Operated 11/28/42

Period number	Date	Calcium				Phosphorus				Nitrogen				Body weight		Urinary citrate	Urinary 17-ketosteroids	Serum				Treatment
		Urinary	Fecal	Intake	Balance	Urinary	Fecal	Intake	Balance	Urinary	Intake	Balance	Theoretical balance	Measured*	Theoretical			Day of period	Calcium	Phosphorus	Alkaline phosphatase	Estradiol benzoate (i.m.)
		mgm. per 24 hr.				*mgm. per 24 hr.*				*grams per 24 hr.*				*kgm.*		*mgm. per 24 hr.*			*mgm. per 100 ml.*		*B.U.*	
1	1/ 5 to 7/43	235	192	173	−254	630	166	805	+ 9	8.90	10.87	+0.88	+2.04		54.00	1045	6.3	II	11.0	3.8	5.5	None
2	1/ 8 to 10/43	267	192	173	−286	630	166	805	+ 9	9.58	10.87	+0.20	+2.28		53.88	1192	9.4					
3	1/11 to 13/43	247	275	173	−349	623	269	805	− 87	9.67	10.87	+0.11	+1.31		53.75	1120	7.0	II	10.1	3.5	3.6	
4	1/14 to 16/43	239	275	173	−341	588	269	805	− 52	9.27	10.87	+0.51	+1.77	53.69	53.66	1221	10.6					
5	1/17 to 19/43	229	261	173	−317	588	236	805	− 19	9.63	10.87	+0.15	+2.10		53.54	1120	9.0	II	11.4	4.2	4.0	
6	1/20 to 22/43	227	261	173	−315	613	236	805	− 44	9.74	10.87	+0.04	+1.68	53.59	53.41	1150	12.0					
7	1/23 to 25/43	198	278	173	−303	525	258	805	+ 22	8.95	10.87	+0.83	+2.59		53.35	1141	10.0	II	11.0	4.4	5.1	1.66 mgm. daily
8	1/26 to 28/43	171	278	173	−276	515	258	805	+ 32	8.83	10.87	+0.95	+2.55	53.63	53.30	1550	9.7					
9	1/29 to 31/43	158	254	173	−239	562	269	805	− 26	9.29	10.87	+0.49	+1.41		53.21	1800	7.7	II	11.0	4.0	3.5	
10	2/ 1 to 3/43	152	254	173	−233	519	269	805	+ 17	9.43	10.87	+0.35	+2.01	53.92	53.11	1765	6.3					
11	2/ 4 to 7/43	148	265	173	−240	528	295	805	− 18	10.49	10.87	−0.71	+1.53		52.91	2122	7.3	II	10.8	4.2	5.8	
12	2/ 8 to 10/43	121	265	173	−213	695	295	805	−185	11.07	10.87	−1.29	−1.17	53.60	52.66		10.0					
13	2/11 to 13/43	127	251	173	−205	668	269	805	−132	10.82	10.87	−1.04	−0.45		52.43	1691	10.7	I	10.7	4.3	5.2	None
14	2/14 to 16/43	148	251	173	−226	680	269	805	−144	11.36	10.87	−1.58	−0.47		52.16	1555	11.0					
15	2/17 to 19/43	143	230	173	−200	694	257	805	−146	11.51	10.87	−1.73	−0.69		51.87	1275	10.3	I	11.3	2.9	5.6	
16	2/20 to 22/43	155	230	173	−212	697	257	805	−149	11.66	10.87	−1.88	−0.65		51.57		12.7					
17	2/23 to 25/43	155	177	173	−159	608	169	805	−128	10.44	10.87	−0.66	+1.62		51.38	1151	10.7	I	10.7	3.6	4.0	
18	2/26 to 28/43	157	177	173	−161	489	169	805	+147	8.78	10.87	+1.00	+3.41	51.33	51.33	1272	10.3	III	11.9	4.6	3.9	

Dietary intake of periods 1 to 18 in amounts per 24 hours: protein (analyzed nitrogen × 6.25) = 67.9 grams, fat (estimated from tables) = 57.2 grams, carbohydrate (estimated from tables) = 210.1 grams, calories (calculated from the values 4 for 1 gram of protein, 9 for 1 gram of fat, and 4 for 1 gram of carbohydrate) = 1,627.

* Initial weight 54.06 kgm.

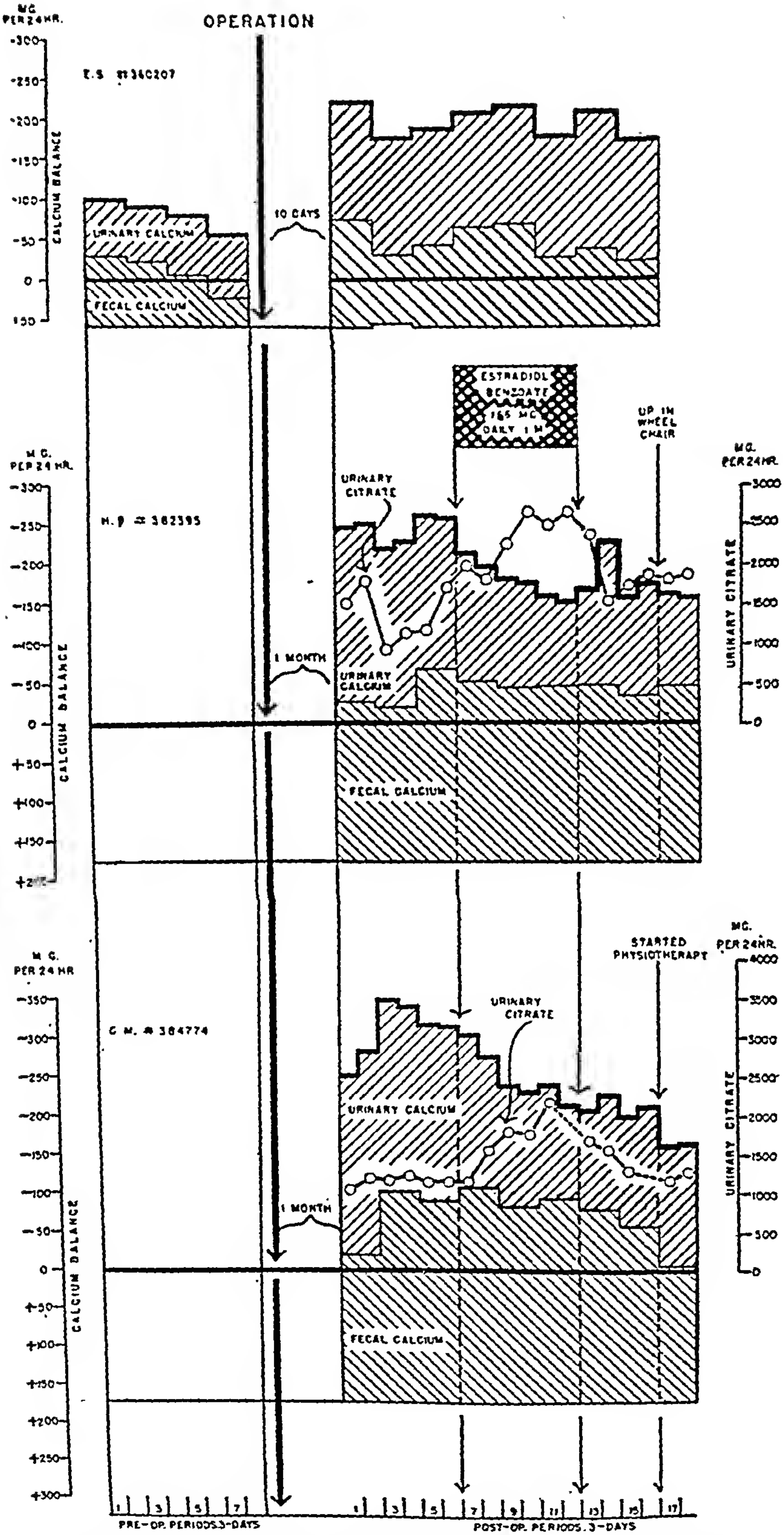

Fig. 10. Metabolic Data for Calcium of Cases 7, 8, and 9. Effect of Estradiol Benzoate as Compared with No Therapy on the Calcium Balances in Patients with Osteoporotic Process due to Operation and Immobilization

For discussion, see text.

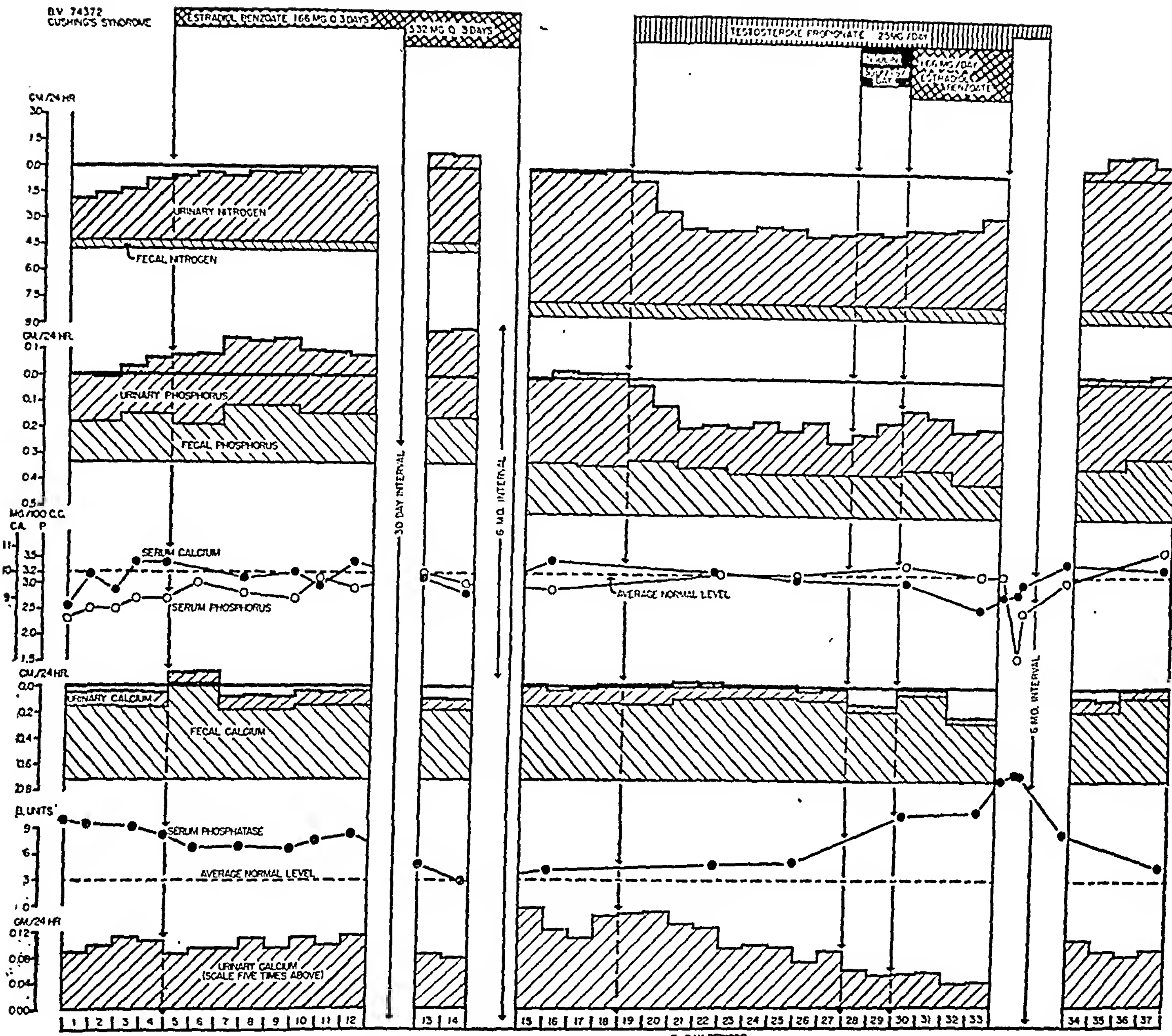

Fig. 11. Case 10 (B. V., M.G.H. 74372): Effect of Estradiol Benzoate and Testosterone Propionate on Nitrogen, Phosphorus, and Calcium Balances, and on Serum Calcium, Phosphorus and Alkaline Phosphatase in a Female Patient with Osteoporosis due to Cushing's Syndrome

For discussion, see text.

At the bottom of the chart, the urinary calcium is shown separately on an enlarged scale.

1 through 33, see (2). The study covers 37 five-day periods obtained on 4 hospital admissions. Two diets were used: one for periods 1 through 14, and a second for periods 15 through 37. The nitrogen intake shown in Figure 11 for periods 17 through 33 is an analyzed value, and differs from that previously published which was taken from a table. The data in Figure 11 are self-explanatory. It should first be noted that the phosphorus balance corresponds reasonably well with the sum of the nitrogen and calcium balances during the last 23 periods, but not the first 14. This suggests some constant error in the first 14 periods, probably the value for the nitrogen intake. A more detailed analysis to emphasize the close agreement between the nitrogen, potassium, phosphorus, and sulphur balances of periods 15 through 33 has already been published (9). Although Albright *et al* (2) concluded from these studies that estrogen was without beneficial effect, this was true with respect to the nitrogen balance but not altogether true with respect to the calcium balance. Thus, with the larger dose of estradiol benzoate in periods 13 to 14 there is an increase, probably significant, in the calcium balance. Further-

more, when estradiol benzoate was added to testosterone propionate therapy in periods 30 through 33, there was a further fall in the urinary calcium excretion and an increase in the positive calcium balance. Other observations to be underlined in Figure 11 are: (*1*) the marked decrease in the urinary nitrogen, phosphorus, and calcium excretions with testosterone propionate therapy; (*2*) the marked rise in the serum phosphatase level when the increase in calcium balance became appreciable (see periods 30 through 33). Whereas Figure 11 suggested that insulin had a marked effect on calcium balance (see periods 28 and 29) the authors are inclined to discount this because of the essentially negative result in a second patient with Cushing's syndrome so treated (18).

Case 11. Cushing's Syndrome with Osteoporosis; Estradiol Benzoate, Testosterone Propionate, and Methyl Testosterone Therapy.

The metabolic data on Case 11 are shown in graphic form in Figure 12. For data in tabular form for periods 1 through 36, see (2). The study covers 55 five-day periods obtained on 6 hospital admissions. The data in Figure 12 are self-explanatory. It should first be noted that the phosphorus balance corresponds reasonably well with the sum of the nitrogen and calcium balances throughout. As in Case 10, one cannot conclude, as did Albright, *et al* (2), that estrogen therapy is without beneficial effect. It was started before the metabolic study was initiated; so its initial effect is hard to evaluate (see periods 1 through 7); however, further studies undertaken 35 days after omitting estrogen show that the calcium balance has changed from positive to negative (compare periods 8 and 9 with 6). Other points to be noted in Figure 12 are: (*1*) the lowering of the urinary nitrogen, phosphorus, and calcium excretions with testosterone propionate therapy (periods 10 through 18, and 23 through 36) and with methyl testosterone therapy (periods 50 through 55); (*2*) the fact that the fecal phosphorus and calcium excretions were also lowered with these 2 testosterone compounds; (*3*) the quick rebound in the nitrogen and phosphorus and not the calcium metabolisms on cessation of testosterone propionate therapy (see periods 19 through 22); (*4*) the steady improvement in calcium metabolism with continued administration of testosterone propionate therapy; (*5*) the elevation of the serum phosphatase with improvement in the calcium balance; and (*6*) the rise in the serum phosphorus level following omission of estradiol benzoate therapy in period 6. The marked improvement in calcium balance in periods 29 through 36 is probably to be attributed to continued testosterone propionate therapy, but the initiation of vitamin D therapy in period 29 makes the exact interpretation difficult. Dehydroisoandrosterone acetate in periods 42 to 46 did not prevent the rebound in nitrogen and phosphorus metabolisms from omission of testosterone propionate therapy.

Case 12. Cushing's Syndrome with Osteoporosis; Progesterone and Testosterone Propionate Therapy.

The metabolic data of Case 12 are shown in graphic form in Figure 13. For data in tabular form, see (2). The study, conducted in 5-day periods, consisted of: (*1*) five control periods; (*2*) seven periods on progesterone therapy, 25 mgm. per day; and (*3*) four periods on testosterone propionate therapy, 25 mgm. intramuscularly per day.

The data in Figure 13 are self-explanatory. As pointed out by Albright, *et al* (2), the progesterone therapy, if anything, had a slightly beneficial effect on nitrogen, phosphorus, and calcium. The effect was not nearly so marked as that obtained in periods 13 to 16 with testosterone propionate therapy. Of interest is the rise in the alkaline phosphatase level in period 16, when the calcium balance became appreciable. It should be noted that the 17-ketosteroid excretion was not lowered by progesterone or elevated by testosterone propionate; the latter finding is surprising, and not in agreement with other studies.

CERTAIN THERAPEUTIC ASPECTS CONCERNING POST-MENOPAUSAL OSTEOPOROSIS

A large number of cases, many complicated by fractures, have been treated with estrogens alone and in combination with testosterone compounds during the past 5 years. As a group, these patients have responded very satisfactorily. Within weeks to months, the pain in the spine and other bones usually has been considerably or completely eliminated. There has frequently been an increase in weight, apparently an increase in the thickness of the skin and an improvement in the general well-being Whereas the study is impossible to control, we have the impression that fractures, especially of the hip, in old ladies have responded better than they would have otherwise. However, in spite of these favorable clinical manifestations, it has been difficult to produce undisputed evidence that the bones (excluding fracture-sites) as visualized by x-ray have become more calcified than before the therapy was instituted. Nevertheless, the recent films of several of the longest-treated cases are fairly convincing.

Dosages have ranged as follows: diethylstilbestrol 0.5 to 1 mgm. daily p.o., estrone sulfate [6] 2.50 to 3.75 mgm. daily p.o., estradiol benzoate 1.66 to 3.32 mgm. 3 times a week i.m., and estradiol dipropionate 5 mgm. weekly i.m. A few patients have been treated by implantation of pellets. Excessive estrogenic effect on the endometrium has been controlled whenever a responsive uterus was present, by interrupting the estrogenic therapy periodically (every 4 to 6 weeks

[6] Conjugated equine estrogens (Premarin [Ayerst, McKenna and Harrison]).

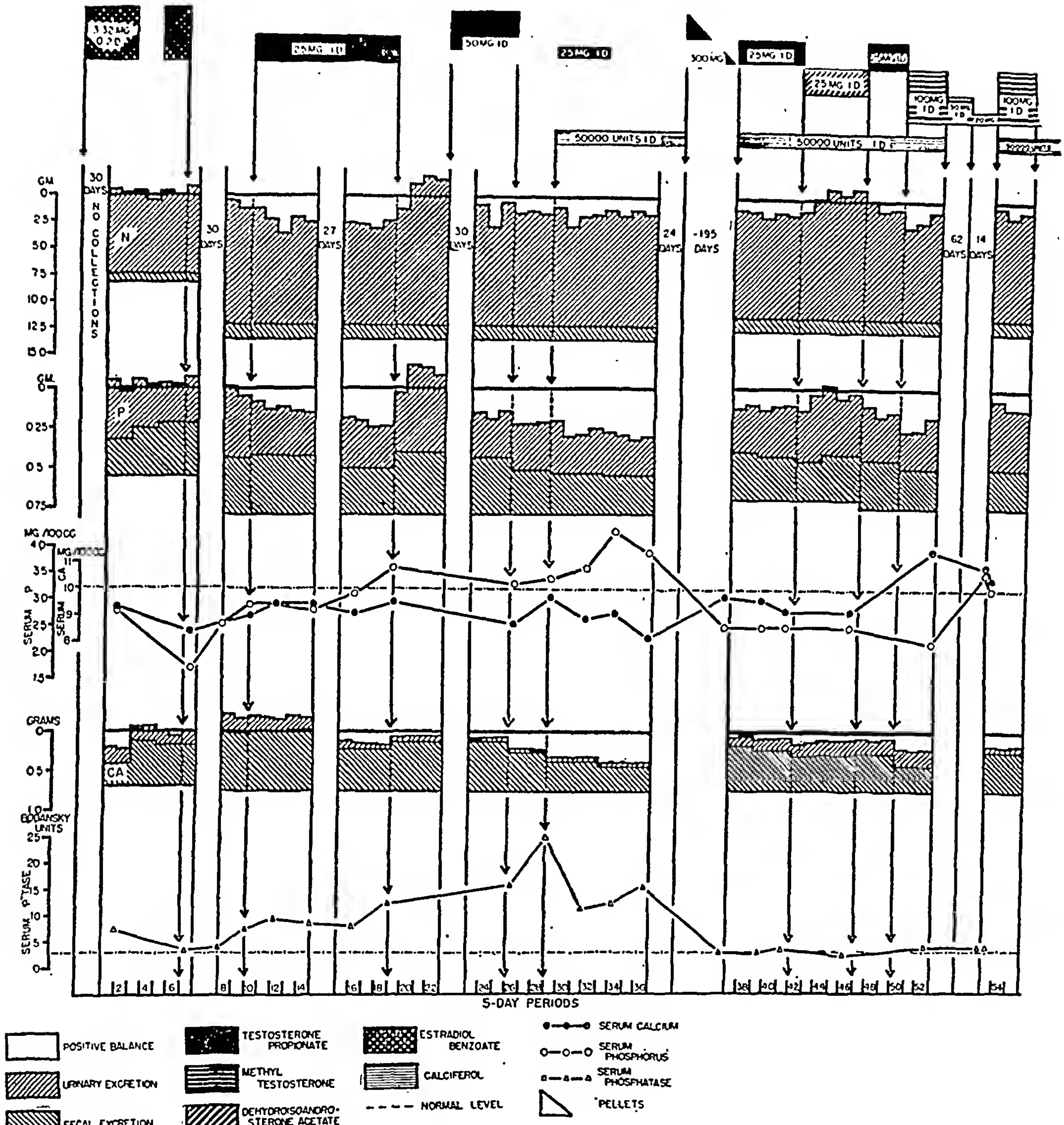

FIG. 12. CASE 11 (R. B., M.G.H. 3397): EFFECT OF ESTRADIOL BENZOATE, TESTOSTERONE PROPIONATE AND METHYL TESTOSTERONE ON NITROGEN, PHOSPHORUS, AND CALCIUM BALANCES; AND ON SERUM CALCIUM, PHOSPHORUS, AND ALKALINE PHOSPHATASE IN A FEMALE PATIENT WITH OSTEOPOROSIS DUE TO CUSHING'S SYNDROME

For discussion, see text.

for 1 to 2 weeks), or by administering at regular intervals (every 4 to 6 weeks) a course of progesterone (5 mgm. daily i.m. for 5 days) or of anhydro-hydroxyprogesterone (40 to 60 mgm. daily p.o. for 5 days). Testosterone compounds cannot be given in most patients with the impunity suggested from Case 4; she was remarkably free from the masculinizing effect of such medication. Most women will not tolerate more than 300 mgm. per month of androgen. We have given methyl

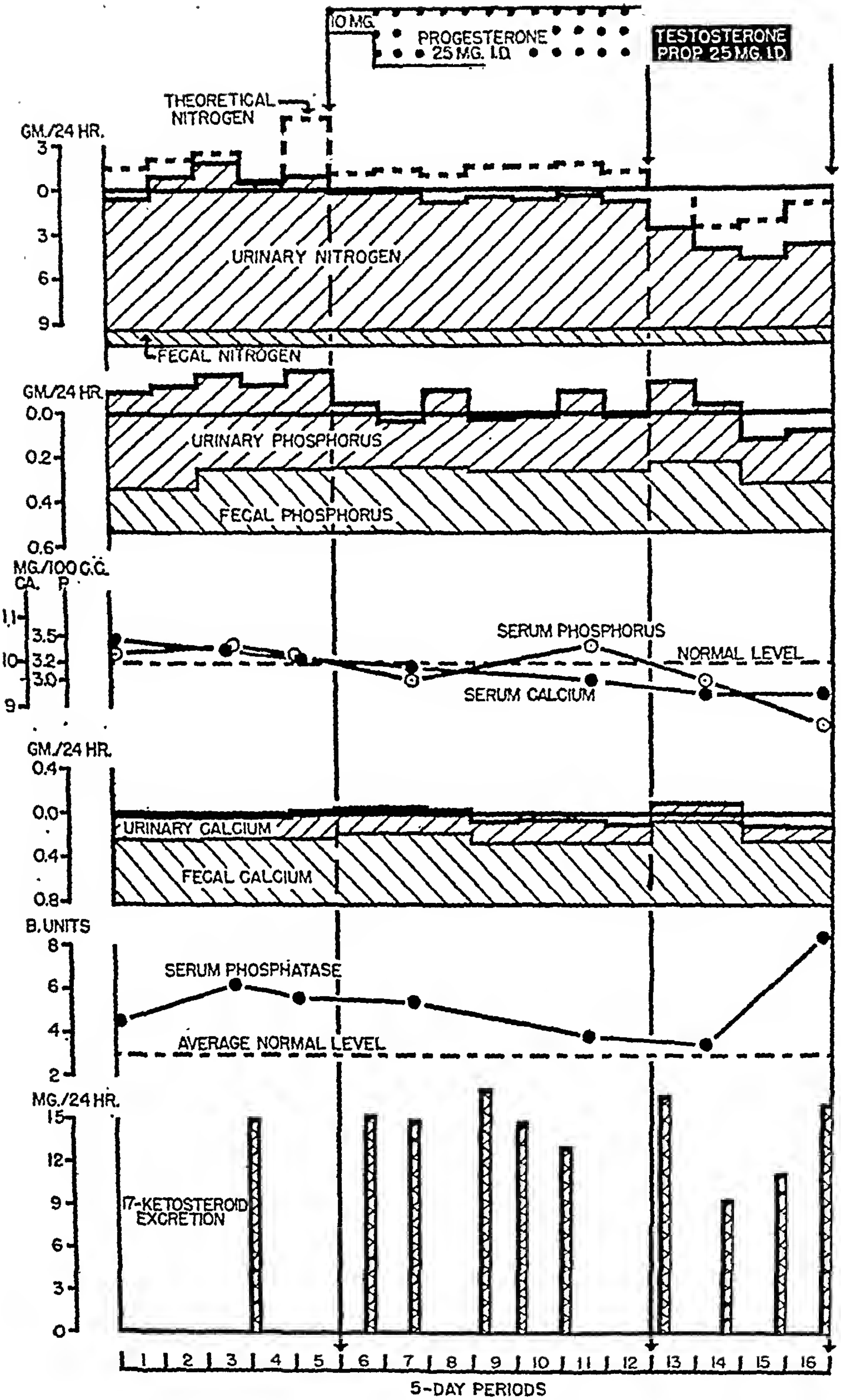

Fig. 13. Case 12 (B. A., M.G.H. 234190): Effect of Progesterone and Testosterone Propionate on Nitrogen, Phosphorus, and Calcium Balances; on Serum Calcium, Phosphorus, and Alkaline Phosphatase; and on Urinary 17-Ketosteroid Excretion in a Female Patient with Osteoporosis due to Cushing's Syndrome

For discussion, see text.

testosterone 10 to 20 mgm. daily p.o., and testosterone propionate 10 to 20 or 25 mgm. a week i.m. One of the most successful methods of administering testosterone compounds to these patients is to implant one or two pellets of testosterone (75 mgm. each [Schering]) every 3 to 4 months. We usually give some form of testosterone at least for the first 6 to 12 weeks.

Since many of the steroids cause sodium retention, the above endocrine therapy may cause edema in certain elderly patients, especially if they have low serum protein levels. If this is not controlled by a low sodium chloride diet, and/or ammonium chloride, the steroid therapy may have to be modified.

Because of the possible danger that continued estrogenic medication may lead to cancer, it has been our practice to interrupt the medication for 7 to 14 days every 4 to 6 weeks, even though the uterus is out. An examination of the vaginal smear every 6 months provides a further safeguard (19). If the uterus is in, a record should be kept of the vaginal bleeding; any bleeding not according to plan (that is, not following estrogen or progesterone withdrawal) should promptly be investigated further.

Since osteoporosis is a deficiency in bone matrix protoplasm, a high protein diet is probably indicated; since it is not a disease of calcium and phosphorus metabolism, excessively high intakes of these minerals and of vitamin D are probably not indicated. Prolonged immobilization should, of course, be avoided if possible, because of the danger of superimposed atrophy of disuse.

SUMMARY

1. Osteoporosis is defined as that form of undermineralization of bone in which the primary defect is a hypofunction of the osteoblasts in laying down bone matrix; eight etiological subgroups are listed.

2. The effect of certain steroid hormones (notably estrogens, androgens, and progesterone) has been studied in 11 cases of osteoporosis: 5 cases of the post-menopausal type, 1 case of the senile type, 2 cases of the type seen following orthopedic operations (atrophy of disuse), and 3 cases of the Cushing's syndrome type.

3. Estrogens in the 2 forms used (estradiol benzoate and diethylstilbestrol) decreased the calcium and phosphorus excretions in the 4 types of osteoporosis studied. Additional observations on estrogen therapy follow.

a. The fecal as well as the urinary calcium and phosphorus excretions were decreased in most instances.
b. The effects were usually manifested within 6 days; did not reach a maximum until after 30 days; and persisted for 30 to 50 days after cessation of therapy.
c. The synthetic estrogen, diethylstilbestrol, appeared to be as effective as the naturally-occurring estrogen, estradiol.
d. The ranges of dosages employed were for estradiol benzoate 3.32 mgm. daily to 1.66 mgm. every 3 days intramuscularly, and for diethylstilbestrol 1 to 15 mgm. daily by mouth. There was no convincing evidence that the larger doses of estradiol benzoate were more effective than the smaller; in one instance (Figure 3) 3.32 mgm. seemed less effective than 1.66 mgm. every third day. In the one case studied, 15 mgm. of diethylstilbestrol daily was probably more effective than 1 mgm. daily.
e. The serum phosphorus levels, which tend to be high in the post-menopausal group, fell in almost all instances.
f. The serum alkaline phosphatase levels, contrary to expectations, did not rise.
g. The urinary nitrogen excretion showed a poorly-sustained decrease.
h. The urinary 17-ketosteroid excretion showed a moderate decrease with estradiol.

4. Androgens in the 2 forms used (testosterone propionate and methyl testosterone) likewise decreased the calcium and phosphorus excretions in the 3 types of osteoporosis (post-menopausal, senile, and Cushing's syndrome) studied. Additional observations on androgen therapy follow.

a. As in the case of estrogens, the fecal as well as the urinary calcium and phosphorus excretions were decreased; the effect of the therapy on the calcium metabolism was slow in reaching its maximum, and persisted for a long time after cessation of therapy; the serum phosphorus levels tended to fall; the

serum alkaline phosphatase levels failed to rise except in the three cases of Cushing's syndrome.

b. In contrast to estrogens, the decrease in the urinary nitrogen excretion was marked and prolonged.

c. The ranges of dosages employed were for testosterone propionate 25 to 50 mgm. daily intramuscularly, and for methyl testosterone 40 to 100 mgm. daily by mouth.

d. Methyl testosterone appeared to be as effective as testosterone propionate.

5. Progesterone, in the dosages of 10, 25, and 100 mgm. daily, had no definite effect whether given alone or in combination with estrogen.

6. The effect on the calcum metabolism of estrogen and androgen in combination was greater than that of either alone in the post-menopausal and senile groups.

7. In Cushing's syndrome estrogen probably does have a beneficial effect on the calcium balance, previous statements to the contrary from this clinic notwithstanding! However, testosterone compounds have a much more striking effect in this condition, as opposed to other types of osteoporosis.

8. The data contain observations on the effect of pregnenolone and dehydroisoandrosterone acetate.

9. A short discussion of certain therapeutic aspects of post-menopausal osteoporosis is included.

The authors are grateful to Drs. Max Gilbert and Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, for generous supplies of estradiol benzoate (Progynon-B), estradiol dipropionate (Progynon-DP), testosterone propionate (Oreton), methyl testosterone (Oreton-M), progesterone (Proluton), anhydro-hydroxyprogesterone (Pranone), dehydroisoandrosterone acetate, pregnenolone, and other steroids.

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APPENDIX

Case histories

Case 1. F. F. (M.G.H. 156453), a 42-year-old woman, had a bilateral oophorectomy at the age of 41 for endometriosis; following the operation she had "nocturnal seizures," the exact nature of which was not determined. During the following year there was a gradual onset of back pain with increasing dorsal kyphosis and a loss of energy. On admission one year after operation, the patient was in good physical condition except for the deformities of her spine; her blood pressure was 130/80. X-rays revealed typical codfish deformity of many of the dorsal and lumbar vertebrae, a collapse of some vertebrae, and anterior wedging of others. Laboratory studies: serum calcium 10.5 mgm. per cent, serum phosphorus 4.2 mgm. per cent, serum alkaline phosphatase 3.6 Bodansky units, serum total protein 7.3 grams per cent, normal glucose tolerance test, some hypoglycemia unresponsiveness in an insulin tolerance test, basal metabolic rate of minus 6, follicle-stimulating hormone test positive for 25 mouse units per 100 ml., and 17-ketosteroid excretion of 4.3 mgm. per 24 hours. This case was mentioned in previous communications (1 [Case 1], 3 [Case 37], 20 [Case 82], 21).

Case 2. E. P. (M.G.H. 203540), a 60-year-old patient, had a physiological menopause at 53. Thirteen months before admission she fell down 6 steps and fractured her first lumbar vertebra; she was kept in bed 5 months for this injury, and then allowed up with a brace. Eight months before admission the 9th dorsal vertebra collapsed. Except for back and chest pain, the patient had no complaints, and was in good general health upon admission. Her blood pressure was 120/90. X-ray examination revealed the fractures of the first lumbar and the 9th dorsal vertebrae, marked osteoporosis of the spine and pelvis, but not of the skull, and gall stones. Laboratory studies: serum calcium 10.1 mgm. per cent; serum phosphorus 3.5 mgm. per cent; serum alkaline phosphatase 3.7 Bodansky units; serum total protein 7.6 grams per cent; no Bence-Jones protein in the urine. This case was mentioned in previous communications (1 [Case 2], 3 [Case 13], 20 [Case 85], 21).

Case 3. A. M. R. (M.G.H. 29358), a 60-year-old physician, developed menopause at 45 following radium treatment of submucous fibroids. Four years before admission she experienced pain in the back while trying to raise a window, and in the ensuing 4 years developed several fractures of vertebrae and progressive deformity of the spine. Physical examination on admission revealed the deformity of the spine and otherwise no abnormalities. Her blood pressure was 148/90. X-ray examination showed deformities of several thoracic and the first lumbar vertebrae, and osteoporosis of the bones of the spine and pelvis but not of the skull. Laboratory studies: serum calcium 10.1 mgm. per cent; serum phosphorus 3.0 mgm. per cent; serum phosphatase 3.7 Bodansky units; serum total protein 6.3 grams per cent. This case has been mentioned in previous communications (1 [Case 3], 3 [Case 32], 20 [Case 84], 21).

Case 4. R. W. (M.G.H. 319940), a 56-year-old woman, had a cholecystectomy at 26, and thyroidectomy for thyrotoxicosis at 46. At 48, an artificial menopause was induced with radium for metropathia hemorrhagica. Three years before admission the patient strained her back opening a heavy window, and thereafter had several episodes of sharp pain in the back when lifting. Physical examination showed a nervous woman with a tremor of her head, and considerable deformity of her back. Her blood pressure was 115/75. X-ray examination revealed extensive osteoporosis with multiple fractured vertebrae; bones of skull were approximately normal in density. Laboratory studies: no abnormalities of the urine, stools, or blood cells; urine calcium 2 to 4 plus by the Sulkowitch test; serum calcium 10.6 mgm. per cent; serum phosphorus 3.1 mgm. per cent; serum alkaline phosphatase 3.7 Bodansky units; serum chloride 93.2 m.eq. per l.; serum carbon dioxide combining power 28.1 m.eq. per l.; non-protein nitrogen level 26 mgm. per cent; and total protein 7.8 grams per cent with an albumin/globulin ratio of 1.7. Electrocardiographic tracing was normal; follicle-stimulating hormone excretion in the urine was high (consistent with the menopause). This case has been mentioned in a previous communication (21).

Case 5. S. B. (M.G.H. 430664), a 58-year-old woman, had at the age of 28 a bilateral oophorectomy with a hysterectomy for pelvic lacerations following childbirth. For some years she had occasional hot flashes and attacks of palpitation and nervousness. At the age of 50 she began to notice weakness and the gradual onset of skeletal deformities involving the skull, shoulder girdle, lower ribs, pelvis, and bones of the legs. At 54 she had acute tonsillitis, and then a tonsillectomy. At 57 she had pneumonia, and after 3 weeks in bed, increased weakness and pain in her tibiae. About this time she used braces on her legs because of difficulty in walking. Shortly afterward she developed low-back pain on weight-bearing.

On admission, the patient was undernourished and deformed with atrophic skin and muscles, dorsal kyphosis and right cervical-dorsal scoliosis, enlarged parietal bosses, bowing of the femora and tibiae, and collapse of the lumbar spine so that the ribs touched the wings of the iliae. The chest was distorted; veins of the neck were distended; cor pulmonale was present; blood pressure was 156/80.

X-rays of the skull, shoulder girdle, lower ribs, pelvis, femora, tibiae, and entire thoracic and lumbar spine except for the upper three dorsal vertebrae showed Paget's disease; in addition there were marked generalized decreased density of bones and typical codfish deformity of many vertebrae. There were pulmonary fibrosis, cardiac enlargement and displacement, and tortuosity of the aorta. Laboratory studies: serum calcium 10.5 mgm. per cent, serum phosphorus 4.2 mgm. per cent, serum alkaline phosphatase 34.3 Bodansky units, serum total protein 7.3 grams per cent, serum non-protein nitrogen 31 mgm. per cent, serum sodium 140.0 m.eq. per l., serum potassium 4.7 m.eq. per l., serum chloride 101 m.eq. per l., serum carbon dioxide content 34.2 m.eq. per l., follicle-stimulating hormone test positive for 192 mouse units per 24 hours, and 17-ketosteroid excretion of 2.6 mgm. per 24 hours. The venous pressure was 65 mm. of water; the vital capacity was 1,200 ml.

Case 6. M. H. (M.G.H. 278511), a male of 72 years, developed pain in the back after a minor injury 1 year before admission (1–1–41). The symptoms persisted in spite of local therapy, and he was referred to the hospital. The only abnormal findings on physical examination were a thin skin and deformities of the spine; his blood pressure was 140/80. X-ray examination of the spine showed marked decrease in density of the vertebrae with a codfish deformity of some, and wedging or collapse of others. Laboratory studies: serum calcium 10.0 mgm. per cent; serum phosphorus 3.1 mgm. per cent; serum alkaline phosphatase 4.2 Bodansky units; serum total protein 7.0 grams per cent; non-protein nitrogen 18 mgm. per cent; urinary 17-ketosteroid excretion 7.2 and 6.9 mgm. per 24 hours; follicle-stimulating hormone excretion in the urine normal; gastric acidity normal. The normal level of the follicle-stimulating hormone excretion is evidence against the idea of the osteoporosis having been due to the "male menopause." This case has been mentioned in previous communications (6, 9, 21).

Case 7. E. S. (M.G.H. 360207), a female of 35 years, had poliomyelitis at the age of 9 involving the left leg alone, and since the age of 14 had worn a 6-pound brace on the left leg. She had always been very active. For the 10 years prior to study she had had metatarsal pain in the right foot, and for 3 years had turned her right ankle frequently. She was admitted for a triple arthrodesis and muscle transplant to strengthen the right ankle. The menstrual history was normal. From the point of view of the experiment the patient can be considered a normal adult female in every respect, except for the residuals of the poliomyelitis of the left leg; her blood pressure was 120/80. Laboratory studies: serum calcium 9.8 mgm. per cent; serum phosphorus 3.5 mgm. per cent; serum alkaline phosphatase 2.4 Bodansky units; and serum total protein 4.7 grams per cent; urinary 17-ketosteroid excretion 7.6 mgm. per 24 hours. This case has been mentioned briefly elsewhere (22).

Case 8. H. D. (M.G.H. 382395), a male fireman of 50 years, fell 3 stories and suffered fractures of ribs, pelvis, right tibia and right fibula, and multiple contusions and abrasions. The patient was in shock on admission, but responded promptly to a blood transfusion. On physical examination he was found to be a well-preserved man without organic disease; blood pressure was 110/60. A Kirschner wire was inserted through the os calcis and a Zimmer bow applied. During the next 2 weeks the fractures were reduced by traction and by several manipulations under anesthesia. The patient was transferred to the metabolic ward where studies were begun 44 days after the accident. Laboratory studies: serum calcium 10.7 mgm. per cent; serum phosphorus 3.3 mgm. per cent; serum alkaline phosphatase 2.7 Bodansky units; serum total protein 6.7 grams per cent. This case has been mentioned briefly elsewhere (23).

Case 9. C. M. (M.G.H. 348774), a male of 24 years, sustained a fracture of the pelvis and of the right femur in an automobile accident 9 months before study. The fe-

mur failed to unite properly and, although the patient was active and able to walk about with a cane, he had unusual motion and instability in his right femur because of the poor union. He was readmitted for bone grafting. Physical examination revealed a young adult male who was normal in all respects except for the incomplete union of his right femur; his blood pressure was 105/60. Laboratory studies: serum calcium 10.3 mgm. per cent; serum phosphorus 4.5 mgm. per cent; serum alkaline phosphatase 2.9 Bodansky units, and serum total protein 6.0 grams per cent. This case has been mentioned briefly elsewhere (24).

Case 10. B. V. (M.G.H. 74372), a female of 25 years, with Cushing's syndrome of 5 years duration. The case history of this patient has been published elsewhere (2 [Case 1]). This case has been mentioned also in other previous communications (6, 9, 20 [Case 37]).

Case 11. R. B. (M.G.H. 3397), a female of 50 years, with Cushing's syndrome of 5 years duration. The case history of this patient has been published elsewhere (2 [Case 2]). This case has been mentioned also in other previous communications (6, 9, 20 [Case 36], 25 [Case 2]).

Case 12. B .A. (M.G.H. 234190), a female of 43 years, with Cushing's syndrome of 6 years duration. A complete case history with autopsy findings is reported elsewhere (26). This case has also been mentioned in previous communications (2 [Case 3], 20 [Case 38]).

BIBLIOGRAPHY

1. Albright, F., Bloomberg, E., and Smith, P. H., Postmenopausal osteoporosis. Tr. Assoc. Am. Physicians, 1940, 55, 298.
2. Albright, F., Parson, W., and Bloomberg, E., Cushing's syndrome interpreted as hyperadrenocorticism leading to hypergluconeogenesis; results of treatment with testosterone propionate. J. Clin. Endocrinol., 1941, 1, 375.
3. Albright, F., Smith, P. H., and Richardson, A. M., Post-menopausal osteoporosis: its clinical features. J. A. M. A., 1941, 116, 2465.
4. Reifenstein, E. C., Jr., and Albright, F., Paget's disease: its pathologic physiology and the importance of this in the complications arising from fracture and immobilization. New Eng. J. Med., 1944, 231, 343.
5. Albright, F., Burnett, C. H., Cope, O., and Parson, W., Acute atrophy of bone (osteoporosis) simulating hyperparathyroidism. J. Clin. Endocrinol., 1941, 1, 711.
6. Albright, F., Cushing's syndrome. Its pathological physiology, its relationship to the adrenogenital syndrome, and its connection with the problem of the reaction of the body to injurious agents ("alarm reaction" of Selye). The Harvey Lecture Series, 1942–1943, 38, 123.
7. Selye, H., The alarm reaction. Cyclopedia Med. Surg. and Spec., F. A. Davis Company, Philadelphia, 1940, 15. Also, Selye, H., The general adaptation syndrome and the diseases of adaptation. J. Clin. Endocrinol., 1946, 6, 117.
8. Reifenstein, E. C., Jr., Kinsell, L. W., and Albright, F., Observations on the use of the serum phosphorus level as an index of pituitary growth hormone activity; the effect of estrogen therapy in acromegaly. Endocrinol., 1946, 39, 71. Also Conference on Metabolic Aspects of Convalescence Including Bone and Wound Healing. Trans. of 12th Meeting, February 4–5, 1946, p. 97; distributed by Josiah Macy, Jr. Foundation, New York.
9. Reifenstein, E. C., Jr., Albright, F., and Wells, S. L., The accumulation, interpretation, and presentation of data pertaining to metabolic balances, notably those of calcium, phosphorus, and nitrogen. J. Clin. Endocrinol., 1945, 5, 367.
10. Talbot, N. B., Saltzman, A. H., Wixom, R. L., and Wolfe, J. K., The colorimetric assay of urinary corticosteroid-like substances. J. Biol. Chem., 1945, 160, 535.
11. Klinefelter, H. F., Jr., Albright, F., and Griswold, G. C., Experience with a quantitative test for normal or decreased amounts of follicle stimulating hormone in the urine in endocrinological diagnosis. J. Clin. Endocrinol., 1943, 3, 529.
12. Abels, J. C., and Dobriner, K., Conference on Metabolic Aspects of Convalescence Including Bone and Wound Healing, Transactions of Seventh Meeting, June 9–10, 1944, p. 122; distributed by Josiah Macy, Jr. Foundation, New York.
13. Farquharson, R. F., Salter, W. T., Tibbets, D. M., and Aub, J. C., Studies of calcium and phosphorus metabolism. XII. The effect of the ingestion of acid-producing substances. J. Clin. Invest., 1931, 10, 221.
14. Howard, J. E., Parson, W., and Bigham, R. S., Jr., Studies on fracture convalescence. III. The urinary excretion of calcium and phosphorus. Bull. Johns Hopkins Hosp., 1945, 77, 291.
15. Forbes, A. P., Donaldson, E. C., Reifenstein, E. C., Jr., and Albright, F., The effect of trauma and disease on the urinary 17-ketosteroid excretion in man. J. Clin. Endocrinol. To be published.
16. Browne, J. S. L., Conference on Metabolic Aspects of Convalescence Including Bone and Wound Healing, Transactions of the Fifth Meeting, October 8–9, 1943, p. 91; distributed by Josiah Macy, Jr. Foundation, New York.
17. Shorr, E., Bernheim, A. R., and Taussky, H., The relation of urinary citric acid excretion to the menstrual cycle and the steroidal reproductive hormones. Science, 1942, 95, 606.
18. Reifenstein, E. C., Jr., and Albright, F., Conferences on Metabolic Aspects of Convalescence Including Bone and Wound Healing, Transactions of the Fifth Meeting, October 8–9, 1943, p. 79; distributed by Josiah Macy, Jr. Foundation, New York.
19. Fremont-Smith, M., Graham, R. M., Janzen, L. T., and Meigs, J. V., The vaginal smear in the diag-

nosis of uterine cancer. J. Clin. Endocrinol., 1945, 5, 40.

20. Fraser, R. W., Forbes, A. P., Albright, F., Sulkowitch, H., and Reifenstein, E. C., Jr., Colorimetric assay of 17-ketosteroids in urine; a survey of the use of this test in endocrine investigation, diagnosis, and therapy. J. Clin. Endocrinol., 1941, 1, 234.
21. Reifenstein, E. C., Jr., Albright, F., Parson, W., and Bloomberg, E., The effect of estradiol benzoate, of testosterone propionate, and of combinations of both on post-menopausal osteoporosis and senile osteoporosis. Endocrinol., 1942, 30, S1024.
22. Reifenstein, E. C., Jr., and Albright, F., Conferences on Metabolic Aspects of Convalescence Including Bone and Wound Healing, Transactions of First Meeting, September 11–12, 1942, p. 37; Transactions of Second Meeting, December 11–12, 1942, p. 69 and 96; and Transactions of Fourth Meeting, June 11–12, 1943, p. 77; distributed by Josiah Macy, Jr. Foundation, New York.
23. Reifenstein, E. C., Jr., and Albright, F., Conferences on Metabolic Aspects of Convalescence Including Bone and Wound Healing, Transactions of Third Meeting, March 12–13, 1943, p. 63; and Transactions of Fourth Meeting, June 11–12, 1943, p. 77; distributed by Josiah Macy, Jr. Foundation, New York.
24. Reifenstein, E. C., Jr., and Albright, F., Conferences on Metabolic Aspects of Convalescence Including Bone and Wound Healing, Transactions of Fourth Meeting, June 11–12, 1943, p. 77; distributed by Josiah Macy, Jr. Foundation, New York.
25. Reifenstein, E. C., Jr., Forbes, A. P., Albright, F., Donaldson, E., and Carroll, E., Effect of methyl testosterone on urinary 17-ketosteroids of adrenal origin. J. Clin. Invest., 1945, 24, 416.
26. Albright, F., and MacMahon, H. E., Clinical Pathological Conference. Bull. New Eng. Med. Center, 1941, 3, 35.

IMMUNOLOGIC STUDIES IN INSULIN RESISTANCE. III. MEASUREMENT OF AN INSULIN ANTAGONIST IN THE SERUM OF AN INSULIN-RESISTANT PATIENT BY THE BLOOD SUGAR CURVE METHOD IN MICE

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The detection of an insulin antagonist in the serum of insulin resistant subjects has been attempted on a number of occasions. In certain instances, the excessive quantities of insulin used in the neutralization experiments in animals may explain the failure to obtain evidence of an insulin antagonist. In the few successful experiments which have been done, little information of a quantitative nature has been obtained. Glen and Eaton (1) and Marble, Fernald and Smith (2) were able to demonstrate decreased responsiveness to insulin in rabbits injected with serum from insulin resistant patients. This change in the experimental animal was still demonstrable a week after injection of the serum. In the same year, Banting *et al.* (3) described experiments in which the incidence of hypoglycemic convulsions was reduced in mice injected with mixtures of serum and varying amounts of insulin. The serum was obtained from a patient with schizophrenia who developed insulin resistance during insulin shock therapy. This case is especially interesting because *diabetes was not present,* suggesting that the resistance was directed against prepared insulin only. When the same serum was retested a week later in mice maintained on a high carbohydrate diet, practically no insulin neutralization could be demonstrated. Lerman (4), in studying serum of 5 patients with insulin resistance, obtained evidence for the presence of an insulin antagonist in 2. One of these serums was provided by the writer, and was obtained from a diabetic patient, A. M., the subject of this and previous reports (5, 6). A high degree of allergy to insulin, coupled with marked insulin resistance, combined to make the giving of insulin to this patient both disagreeable and ineffective. A further important feature was the presence of a relatively mild degree of diabetes, making possible prolonged periods during which insulin therapy could be omitted without immediate danger to the patient's life. A number of hospital admissions provided opportunity to show that:

a. At the end of a period of 5 months or more without insulin therapy, small doses of insulin would give rise to urticaria, constriction in the chest, and, on one occasion, collapse.

b. After desensitization with graded doses of insulin, a period of 3 to 5 days followed during which the injection of relatively small doses of insulin would be followed by a fall in blood sugar.

c. Within about 11 to 15 days of starting desensitization and treatment with insulin, a solid resistance to insulin developed.

These changes were correlated with the absence, and then the appearance, of insulin-neutralizing activity in the patient's serum. The allergic reactions appeared to be mediated by a mechanism quite independent of that giving rise to resistance (5).

The method previously used for the demonstration of insulin-neutralizing activity of the patient's serum (6) consisted in observing the incidence of hypoglycemic symptoms in mice injected with a mixture of serum and insulin. This method had a number of disadvantages which outweighed its simplicity. A relatively large dose of insulin was required in order to produce symptoms of hypoglycemia with some regularity when normal serum was used, and because of this the chance of detecting small amounts of neutralizing activity in the serums to be tested was decreased. This, in turn, led to the use of a large amount of serum

in each animal, a circumstance which in itself introduced non-specific blood-sugar raising effects (see below). These effects had to be distinguished from specific insulin neutralization, and tended to decrease the regularity of symptoms in control tests. Furthermore, the recognition of truly hypoglycemic symptoms was not always easy. Finally, reliance on the incidence of hypoglycemic symptoms as an indication of insulin effect provided no direct measure of the effect of the injected mixture on the blood sugar itself. With the present method, some of these difficulties are avoided, and the small amounts of serum required made possible a more detailed study of the behavior of the serums tested. One serum, obtained from A. M. before insulin therapy was started in the patient's final and hitherto unreported hospital admission, will be referred to below as "before" serum, and a second specimen, obtained 25 days after insulin treatment had been started, at a time when resistance had been reestablished, will be referred to as "after" serum.

CASE REPORT

(*Final hospital admission* [1])

After discharge from the Evans Memorial Hospital on 11/15/42, the patient, a 45-year-old, white female, was seen at infrequent intervals. In the fall of 1943, a diagnosis of pulmonary tuberculosis was made, and she spent several months in a sanatorium for tuberculosis. She refused to stay, however, and received no further hospital care until April, 1944, when she was readmitted to the Evans Memorial Hospital with advanced pulmonary tuberculosis. With the exception of this, examination revealed findings similar to previous admissions. No insulin had been given since November, 1942, and it was thought advisable to try insulin therapy again. The patient was intolerant of many procedures, and no attempt was made to restudy the effect of insulin therapy in detail. As shown in Table I, the administration of insulin in daily doses of between 40 and 102 units brought about a marked reduction in the 24-hour output of glucose for a period of 6 days. This was first apparent on the third day of insulin treatment, at which time the blood sugar fell from an average of 300 to 154 mgm. per 100 ml. Some degree of insulin effect was apparent up to the ninth day of therapy. Thereafter no response to insulin occurred with daily doses of 120 to 280 units. All insulin therapy was stopped on 5/20/44 after a total of 3,969 units had been given in a period of 33 days. During the treatment with insulin, the patient complained of itching and soreness at the site of the injections, and, in addition,

[1] Previous hospital admissions have been reported (5).

TABLE I

Insulin dosage and glucose excretion of A. M. during final hospital admission

Day of month	Insulin	Glucose excretion	Blood sugar	Remarks
	units per day	*grams per day*	*mgm. per 100 ml.*	
4/12/44	0	76		
4/13/44	0	93	FBS 314	
4/14/44	0	100		
4/15/44	0	111		
4/16/44	0			
4/17/44	31	77	FBS 520	"Before" blood taken. Urticaria
4/18/44	58	53		Urticaria
4/19/44	96	20	4 PM 154	Urticaria
4/20/44	40	5.1		
4/21/44	82	tr.	FBS 352	
4/22/44	64	tr.	FBS 230	
4/23/44	130	tr.		
4/24/44	120	26		
4/25/44	220	48		
4/26/44	280	81	FBS 520	
4/27/44 to 5/20/44	120 to 280	150 to 211	425 on 5/12/44	"After" blood taken on 5/12/44

had several attacks of urticaria while desensitization was in progress. The sequence of events following attempted therapy with insulin was similar to that following earlier attempts (5), and the presence of active tuberculosis appeared to have no influence on the patient's resistance.

The patient refused sanatorium care, and finally left the hospital against advice. It was learned later that she died at home in the fall of 1944, and that no autopsy was done. The discharge diagnoses were: Diabetes mellitus with diabetic retinitis and peripheral neuritis; insulin allergy and resistance; chronic pyelonephritis; pulmonary tuberculosis.

MATERIALS AND METHODS

Each of 6 large white mice, starved for 18 hours, was injected intra-abdominally with 0.2 ml. of a mixture of serum, diluted or undiluted, and a dilution of insulin, after a fasting blood specimen had first been obtained. Subsequent blood samples were taken 30 minutes and 60 minutes after the injection of the mixture to be tested. For each blood sugar determination, .1 ml. of blood was drawn from a clean cut across a tail vein into a .1 ml.

micro blood sugar pipette which had been rinsed with a heparin solution containing 20 mgm. in 1 ml. of distilled water and thoroughly dried. The presence of heparin reduced the incidence of clotting, and did not interfere with the blood sugar determinations. These were done according to the technic of Folin, and the color readings were made in an Evelyn colorimeter. No animal was used more than once. The flow of blood was found to be freer when the animals were kept warm during the test. This was done with an ordinary desk lamp held at such a distance from the animals that a temperature of 27° C. to 30° C. was maintained. The performance of the test was facilitated by first drawing up a schedule in which the times at which blood samples were to be obtained and injections given were noted. A period of 5 minutes was allowed for each procedure.

Serum for testing was obtained from clotted blood, drawn with sterile precautions, and was stored in the frozen state at −10° C. The "before" and "after" serums from A. M., and serum from a case of uncomplicated diabetes, all of which contained high concentrations of glucose, were dialyzed in 10 ml. amounts in cellophane bags against 40 ml. of buffered salt solution at pH 7.4 for 18 hours at 4° C. No significant change in volume occurred.

TABLE II

Changes in the blood sugar of mice following the injection of normal serum and "before" serum from A. M., with and without insulin

Test no.	Serum	Insulin	Mouse no.	FBS	Change in blood sugar at		Mouse no.	FBS	Change in blood sugar at		Average change in blood sugar at	
					30 min.	60 min.			30 min.	60 min.	30 min.	60 min.
		units per mouse		*mgm. per 100 ml.*				*mgm. per 100 ml.*				
1	Normal	0	1 2 3	116 95 125	+4 +22 +13	+4 +28 +5	4 5 6	80 128 102	+28 +43 +37	+31 +35 +31	+24	+22
2	Normal	0	1 2 3	87 79 113	+28 +3	+11 +50 +20	4 5 6	107 124 	+3 −9 	0 −1 	+6*	+16**
3	Uncomplicated diabetes	0	1 2 3	116 95 125	+4 +22 +13	+4 +28 +5	4 5 6	80 128 102	+28 +43 +37	+31 +35 +31	+24	+22
4	A. M. "before"	0	1 2 3	113 92 112	+38 +39 +45	+63 +55 +42	4 5 6	125 88 132	+18 +41 +33	+21 +48 +39	+35	+45
5	A. M. "after"	0	1 2 3	91 94 89	+16 +27 +25	+9 +30 +40	4 5 6	106 81 102	+27 +17 +38	+43 +21 +40	+25	+30
6	A. M. "after"	0	1 2 3	83 108 63	+42 +29 +22	+72 +24 +22	4 5 6	91 70 88	+43 +43 +48	+81 +40 +56	+38	+49
7	Normal	.005	1 2 3	97 95 86	−5 −17 +10	−15 −21 −10	4 5 6	86 92 114	−11 −5 −26	−10 −6 −36	−9	−16
8	A. M. "before"	.005	1 2 3	94 98 91	−44 −5 −10	−46 −24 −26	4 5 6	83 91 90	+3 −14 −7	−14 −17 −16	−13	−24
9	Uncomplicated diabetes	.005	1 2 3	94 83 88	+3 −20 −26	−12 −16 −27	4 5 6	81 104 109	−15 −36 −15	−18 −43 −39	−18	−26
10	Normal	.01	1 2 3	90 97 106	−19 −13 −18	−24 −38 −22	4 5 6	110 89 92	−28 −16 −15	−32 −31 −32	−18	−30
11	A. M. "before"	.01	1 2 3	97 110 79	−53 −45 −25	−63 −67 	4 5 6	92 112 150	−51 −61 −99	−48 −79 −85	−55	−68**

* Average based on 4 animals.

** Average based on 5 animals.

Glucose determinations were made on the dialyzed serums, as well as other serums which were not dialyzed, and all serums tested contained between 40 and 55 mgm. glucose per 100 ml. Dialysis produced no observable change in the behavior of serums in mice, other than that attributable to the reduction in the glucose content.

RESULTS

Six tests in which mice were injected with mixtures of a serum and saline with no added insulin are shown in Table II (tests 1 to 6). Of the 35 mice tested, all but 1 showed a rise in blood sugar following injection. The rise was greatest in the first 30-minute period. Wide variations occurred in the responses of individual mice and, with the small number of animals used, the difference in the degree of the rise from test to test is hardly susceptible of interpretation. These results stand in sharp contrast to those obtained when insulin was injected with normal serum, serum from an uncomplicated case of diabetes mellitus, or "before" serum from A. M. A moderate but prompt fall in blood sugar followed the injection of .005 unit of insulin mixed with serum in 15 of 18 mice, and this fall was sustained or more marked at 60 minutes (Table II, tests 7 to 9). A greater fall occurred in mice receiving .01 unit (Table II, tests 10 and 11).

Tests with "after" serum from A. M., in which varying amounts of insulin were added to a constant volume of serum, are shown in Table III. In all tests with .06 unit or less, a rise in the average blood sugar occurred at the end of 30 minutes. Only in test 9 did a fall occur in the first 30 minutes, the amount of insulin required to cause this, .075 unit, being 15 times greater than that required when a normal or "before" serum was used. A rise in blood sugar over the fasting level was also observed at the end of 60 minutes in 2 tests with .01 unit, 1 of 2 tests with .02 unit, and 2 tests with .03 unit. The fall in test number 3 is unexplained. With .05 and .06 units, a moderate fall was seen at 60 minutes. In the test with .075 unit, the fall in the blood sugar was of about the same degree as that seen with "before" and normal serums in combination with .005 unit of insulin.

Titration of the neutralizing activity of this serum was also attempted by mixing varying dilutions of serum with a small constant dose of insulin. The results are shown in Table IV. A rise

TABLE III

Changes in the blood sugar of mice following the injection of a mixture of "after" serum with varying amounts of insulin

Test no.	Insulin	Mouse no.	FBS	Change in		Mouse no.	FBS	Change in		Average change in	
				30 min.	60 min.			30 min.	60 min.	30 min.	60 min.
	units per mouse		*mgm. per 100 ml.*				*mgm. per 100 ml.*				
1	.01	1	100	−1	−1	4	99	+20	+5	+10	+9
		2	95	+7	0	5	97	+20	+20		
		3	95	+1	+3	6	125	+13	+28		
2	.01	1	133	+3	+16	4	71	+14	+7	+10*	+9*
		2	100	+11	−3	5					
		3	88	+16	+23	6	95	+5	+1		
3	.02	1	92	−38	−41	4	126	−15	−38	+6	−13*
		2	97	0	−23	5	81	+36	+21		
		3	90	+39		6	92	+14	+4		
4	.02	1	64	+23	+15	4	82	+22	+17	+33	+25
		2	88	+46	+41	5	91	+33	+31		
		3	80	+43	+32	6	88	+30	+17		
5	.03	1	88	+22	+2	4	71	+29	+23	+20	+4
		2	81	+29	+11	5	78	+20	−2		
		3	88	+10	−7	6	97	+13	−4		
6	.03	1	92	+12	+4	4	69	+41	+25	+28	+23
		2	82	+19	+34	5	94	+32	+24		
		3	75	+41	+35	6	97	+27	+17		
7	.05	1	95	+5	−16	4	78	+2	−8	+12	−7
		2	88	+30	+8	5	76	+16	−8		
		3	82	+13	−7	6	82	+8	−10		
8	.06	1	84	+22	−13	4	95	+29	−4	+12	−12
		2	102	+22	+18	5	104	+6	−29		
		3	88	−1	−14	6	96	−8	−33		
9	.075	1	89	−14	−21	4	74	−6	−19	−9	−21
		2	77	−9	−17	5	75	+3	−16		
		3	83	−15	−24	6	97	−16	−32		

* Average based on 5 animals.

in the average blood sugar occurred at the end of 30 minutes in tests done with serum diluted 1:3, 1:6, 1:12 and 1:24. When diluted 1:48, a marked fall occurred in 30 minutes. At the end of 60 minutes the results in this group of tests were less regular. A rise occurred in 3 tests (Table IV, tests 1, 3 and 4) with serum diluted 1:3, 1:6, and 1:12, but a fall occurred in 1 test done with 1:6, and 1 done with 1:24. When compared with the results obtained with undiluted normal serum (Table II, tests 6 to 9), these 2 tests suggest that some neutralization of insulin took place. Marked variation in the behavior of individual mice is evident in all but the first and the last.

The effect of injecting .1 ml. of "after" serum 18 hours before the injection of insulin was also tried. The amounts of serum and insulin were such as to make the tests comparable to those done with mixtures of insulin and serum. With a dose of .005 unit per mouse there was a rise in blood

TABLE IV

Changes in the blood sugar of mice following the injection of a mixture of varying dilutions of "after" serum and 0.005 unit insulin

Test no.	Dilution of serum	Mouse no.	FBS	Change in 30 min.	Change in 60 min.	Mouse no.	FBS	Change in 30 min.	Change in 60 min.	Average change in 30 min.	Average change in 60 min.
			mgm. per 100 ml.				*mgm. per 100 ml.*				
1	1:3	1	65	+25	+32	4	89	+21	+9	+25	+26
		2	105	+39	+39	5	110	+15	+35		
		3	96	+47	+43	6	95	+2	+6		
2	1:6	1	89	−12	−15	4	82	+1	+1	+6	−6
		2	75	+13	−9	5	88	−11	−14		
		3	73	+7	+6	6	91	+6	−3		
3	1:6	1	95	+13	+22	4	109	+19	+20	+7	+5
		2	105	+2	0	5	124	−16	−36		
		3	107	+21	+24	6	78	+3	−1		
4	1:12	1	124	+2	−12	4	67	+24	+26	+12	+6
		2	117	+12	−11	5	70	+18	+16		
		3	86	+7	+6	6	88	+12	+14		
5	1:24	1	92	+7	−8	4	102	+5	−5	+6	−5
		2	67	+4	−6	5	77	+16	−6		
		3	78	+13	+2	6	74	−7	−9		
6	1:48	1	93	−39	−46	4	82	−38	−43	−28	−33
		2	75	−21	−22	5	80	−19	−26		
		3	86	−29	−35	6	108	−25	−28		

sugar in 5 of 6 mice in 30 minutes, and in 4 of 6 mice at 60 minutes with an average rise of 11 mgm. per 100 ml. in 30, and of 9 mgm. per 100 ml. in 60 minutes. With .01 unit per mouse there was a rise in all 6 mice at 30 and 60 minutes. However, with .03 unit per mouse, the average change was downward at 30 and 60 minutes. Thus, as would be expected, the anti-insulin effect of the serum was diminished when a period of time was allowed to elapse between the injection of the serum and the administration of insulin. It appears, therefore, that the insulin antagonist in the "after" serum of A. M. is most active when it is injected simultaneously with insulin. This suggests that this antagonist is not the glycotropic factor of the pituitary. This hormone, when injected, produces no immediate effect on the susceptibility of the animal to insulin, but on the contrary, a period of several hours is required before interference with the action of insulin becomes demonstrable (7).

No decrease in the insulin-neutralizing effect of the serum could be demonstrated when insulin was added to the serum in 3 to 6 divided doses at 5-minute intervals in amounts totaling .03 unit per mouse and .06 unit per mouse. This was done in an unsuccessful attempt to demonstrate a Danysz phenomenon.

DISCUSSION

The performance of the test, irrespective of the injection of insulin, may be assumed to have effects on the experimental animal which, in themselves, may cause elevation or depression of the blood sugar. Unexplained falls in blood sugar of individual mice receiving serum or saline without insulin were occasionally seen in some earlier unreported experiments, but were not observed in later experiments. The marked tendency for the blood sugar to rise when serum alone was injected (Table II) may probably be explained as being due to the animal's fright and anger at being held, bled, and injected, and to the non-specific effect of injecting foreign material. The regular rise in the blood sugar of mice following the injection of serum without any added insulin casts some doubt on the significance of experiments which have been carried out in the past, allegedly demonstrating a specific blood-sugar raising effect of serum from an insulin-resistant subject (8).

The results of this study support, in a roughly quantitative manner, the conclusions reached in the earlier qualitative tests (6). An estimation of the insulin inactivating capacity of the patient's plasma volume at the time the "after" serum was drawn readily explains the resistance to insulin and, in addition, indicates that the doses given the patient during the final hospital admission were a fraction of what would have been required to induce a fall in blood sugar. The amount of insulin which, on the basis of the studies made, would be neutralized by the patient's entire plasma volume (2,500 ml.) can be estimated in a number of ways. One figure can be arrived at by using the largest dose of insulin per mouse with which neutralization of insulin was demonstrable, namely .06 unit per .1 ml. (Table III, test 8).

$$.06 \times 10 \times 2{,}500 = 1{,}500 \text{ units}$$

When calculated on the basis of tests in which dilutions of serum were made, the required dose for rapid insulin effect would be (Table IV, test 5)

$$.005 \times 24 = .12 \text{ u per .1 ml. serum}$$

and

$$.12 \times 10 \times 2{,}500 = 3{,}000 \text{ units}$$

In tests of this kind the agreement cannot be expected to be close, but the evidence clearly indicates that something well over 1,000 units would be required in order to bring about a rapid fall in blood sugar in the patient. This reasoning is based on the extremely doubtful assumption that the insulin-neutralizing factor was present in the circulating blood alone. If an antibody was actually involved, the tissues might also have played a large part in the inactivation of the administered insulin. Furthermore, the continuous production of the antibody would serve to augment the dose of insulin required to bring the diabetes under control if the doses were spread, as is usually the case, over a period of time. Thus, the reported instances of insulin-resistant individuals requiring 3,000 to 4,000 units of insulin daily can be explained on the very mechanism that accounted for insulin resistance in A. M.

Assuming the immunologic nature of insulin resistance in A. M., the fact that relatively little insulin could be tolerated because of the high degree of allergy provided a circumstance especially favorable for the demonstration of the insulin-neutralizing antibody. Administration of doses of insulin sufficiently large to have controlled the diabetes should at the same time have brought about a marked diminution in the antibody content of the circulating blood. This may in part explain the failure of many of the attempts in the past to demonstrate insulin-neutralizing activity in the serum of resistant subjects, who, after all, can be recognized clinically only by their failure to respond to any but very large doses of insulin. Inadequate treatment of the diabetes with insulin, or better still, no treatment at all for a brief period before obtaining serum, would probably facilitate the demonstration of insulin-neutralizing activity in the serum of resistant individuals.

The ready demonstration of insulin-neutralizing activity in the serum of resistant patients will probably depend on a high degree of resistance. Thus, patients who are resistant to insulin but who require only 300 to 500 units daily will, in all likelihood, have nothing in their serums that could be demonstrated by the technic described. Study of such patients with a view to determining the cause of the resistance will probably have to depend on different methods.

Failure of the usual *in vitro* immunologic tests for the demonstration of antibody in the serum of insulin-resistant subjects is, like the failure to demonstrate the Danysz effect in the serum of A. M., no argument against the immunologic nature of the resistance. Such methods depend for their success on suitable characteristics of both the antigen and the antibody, as well as adequate concentrations of the latter. The evidence to date (6) is against the view that the precipitins or complement fixing antibody occasionally encountered in the serum of patients receiving insulin (9), or animals injected with insulin in an attempt to induce allergy or antibody formation (10, 11), are actually concerned with insulin resistance.

SUMMARY AND CONCLUSIONS

1. A method is described which adequately demonstrates interference, by serum of an insulin-resistant patient, with the blood sugar lowering effect of insulin in mice, and which permits rough quantitation of this interference.

2. Estimation, on the basis of the results of this study, of the total insulin-neutralizing activity of the patient's blood volume, readily explained the patient's resistance.

3. The mechanism giving rise to insulin resistance in the patient studied, presumably immunologic in nature, can explain the extreme instances of insulin resistance which have been occasionally reported.

BIBLIOGRAPHY

1. Glen, A., and Eaton, J. C., Insulin antagonism. Quart. J. Med., 1938, 7, 271.
2. Marble, A., Fernald, A. T., and Smith, R. M., Effect of human diabetic plasma upon blood sugar curves in rabbits following insulin. Endocrinology, 1940, 26, 735.
3. Banting, F. G., Franks, W. R., and Gairns, S., Anti-insulin activity of serum of insulin-treated patients. Am. J. Psychiat., 1938, 95, 562.
4. Lerman, J., Insulin resistance. The role of immunity in its production. Am. J. M. Sc., 1944, 207, 354.
5. Lowell, F. C., Immunologic studies in insulin resistance. I. Report of a case exhibiting variations in resistance and allergy to insulin. J. Clin. Invest., 1944, 23, 225.
6. Lowell, F. C., Immunologic studies in insulin re-

sistance. II. The presence of a neutralizing factor in the blood exhibiting some characteristics of an antibody. J. Clin. Invest., 1944, 23, 233.
7. Thompson, David L., The anterior lobe of the hypophysis in intermediary metabolism. Glandular Physiology and Therapy: A symposium. American Medical Association, Chicago, 1942, p. 56.
8. Wayburn, E., and Beckh, W., Insulin resistance in diabetes mellitus. J. Clin. Endocrinol., 1942, 2, 511.
9. Goldner, M. G., and Ricketts, H. T., Insulin allergy. A report of eight cases with generalized symptoms. J. Clin. Endocrinol., 1942, 2, 595.
10. Wasserman, P., Broh-Kahn, R. H., and Mirsky, I. A., The antigenic property of insulin. J. Immunol., 1940, 38, 213.
11. Wasserman, P., and Mirsky, A., Immunological identity of insulin from various species. Endocrinology, 1942, 31, 115.

TRICHINELLA SKIN TESTS IN AN ORPHANAGE AND PRISON. COMPARISON WITH SEROLOGIC TESTS FOR TRICHINOSIS AND WITH THE TUBERCULIN REACTION [1]

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The possibility that an unrelated disease process may alter the reaction to the trichinella skin test was uncovered in the course of an investigation on the incidence of trichinosis. A higher percentage of positive skin reactions to trichinella antigen (18.3 per cent) was obtained in 674 patients with active tuberculosis confined in 3 sanatoria, than was found in 335 patients without tuberculosis (7.2 per cent) studied in 2 general hospitals for shorter periods of time (1). On the other hand, the incidence (18.4 per cent) in 825 patients without active tuberculosis confined in 3 hospitals for mental diseases was almost identical with that found in the sanatoria. This finding suggested that some factor connected with residence in an institution might account for the high incidence. An analysis of positive reactors according to the duration of stay in an institution revealed that the incidence of positive reactions reached a peak at 18 to 29 months; no history suggesting subclinical institutional epidemics was elicited (1).

Experiments were undertaken to determine whether positive trichinella skin tests could be produced in the absence of infection by the ingestion of killed trichinae, as in meat rendered non-infectious by cooking inadequate to denature the protein of the parasite. Sensitization was not induced in the animals used for these experiments (2). The possibility of a biologic cross reaction, detectable by skin test, between *Mycobacterium tuberculosis* and *Trichinella spiralis* was investigated in rabbits infected with trichinae and a bovine strain of tubercle bacilli simultaneously. No cross reaction was detected in these rabbits by skin tests (3).

The present experiments were undertaken to determine whether a biologic cross reaction detectable by skin test might be demonstrated in human beings. It was necessary to select subjects for testing who did not have active tuberculosis or acute psychiatric disturbances (1). In order to control adequately the factor of age in institutionalized individuals, it was desirable to choose one institution in which only children were in residence, and one institution where only adults were confined for comparable periods of stay. It had been found that the incidence of positive skin reactions decreases with advancing age, whereas the incidence of infection proved at autopsy increases with advancing age (1). An orphanage and a prison, both under close medical supervision, most nearly met the requirements.

MATERIAL

A total of 526 individuals were subjected to skin tests. Each person had been in residence in the institution for at least one year; the majority had been in residence for 2 to 15 years (Table III). In none was there a history of tuberculosis or of recognized mental disease. No patient was known to have had trichinosis, and there had been no epidemics in either institution which suggested trichinosis.

At the Methodist Children's Home, Winston-Salem, N. C., 285 children, varying in age from 6 to 19 years, were tested. The children are all white and are housed according to age in groups of 25 to 35 in 13 cottages; every 2 years they "graduate" from one cottage to another, so that the group is fairly homogeneous. Though there are kitchens in each cottage, only 3 are currently used to feed groups of boys included in this study. The remainder of the boys and all of the girls eat in a central dining room. Pork obtained from hogs raised on the orphanage farm is served twice weekly from November to February; the children eat little pork and prefer beef, which is also available. Routine chest x-rays are not made, but none of the children found to have a positive tuberculin skin test had clinical or roentgenologic evidence of tuberculosis when examined subsequently.

In the North Carolina Central Prison, Raleigh, N. C., a group of 241 prisoners, varying in age from 16 to 77 years, was studied. The 58 female prisoners are housed

[1] This study was aided by a grant from the John and Mary R. Markle Foundation.

in a separate building with a separate kitchen; hence, this group in effect represents 2 institutions. No prisoner had roentgenologic evidence of pulmonary tuberculosis when a photo-roentgen survey was made a few months prior to the initiation of this study. Pork raised on the prison farm is served once weekly. The quantity of pork eaten at the prison appears to have been slightly greater for the 2 years preceding the test than that eaten at the orphanage.

METHODS

In all instances, a 1:10,000 solution of trichinella extract (Lederle) and a phenolized phosphate buffer saline control solution were used.[2] A 1:1,000 dilution of tuberculin (Lilly) in distilled water and the tuberculin control solution were freshly prepared for use in each institution.

New syringes and needles were obtained for the skin tests, and were not used for any other purpose. One syringe and needle was always used for each test solution, and 2 others for the controls; the syringes and needles were never interchanged. After being used, they were washed with distilled water only, placed in marked tubes, and sterilized in an autoclave.

An area on the flexor surface of both forearms was cleaned with 70 per cent alcohol, and 4 workers simultaneously injected 0.02 ml. of the trichinella test and control solutions in the right arm, and 0.1 ml. of the tuberculin test and control solutions in the left arm. The test solution was always injected proximal to the control solution.

The reactions were read at 30 minutes and 24 hours after injection. The tuberculin reactions were graded by the usual scale from 1 to 4 plus. The trichinella reactions were also recorded on a scale of 1 to 4 plus, 1 plus denoting an immediate reaction which exceeded the diameter of the injected bleb by 5 mm. or more (1).

[2] The trichinella antigen and control solution used in this study were supplied by Lederle Laboratories, Pearl River, N. Y.

Ten weeks after the skin tests were performed, blood serum was drawn from the antecubital vein of the orphans for serologic testing. Serum was obtained from the prisoners 17 to 19 weeks after skin-testing.

Flocculation tests were performed according to the technique described by Suessenguth and Kline (4). The trichinae from which the antigen was prepared were recovered from rabbits fed 8 weeks previously on a rat infected with a strain of trichinella parasites obtained from the National Institute of Health (2). Precipitin reactions were done with a 1:200 dilution of antigen prepared from the same dried trichinae used for the flocculation test.

RESULTS

Skin tests

Orphanage. Fifty-seven children (20.0 per cent) gave positive trichinella skin tests; 51 were immediate reactions, and 9 were delayed reactions which appeared at 24 hours. Three tests were positive at both 30 minutes and 24 hours (Table I). Seven children reacted to both the test and control solution, and were calculated as negative in the statistical analysis. Forty children (14.0 per cent) gave positive tuberculin reactions at 24 hours. The immediate reactions to the tuberculin test and control solution were not thought to be significant. Six children reacted positively to both trichinella extract and tuberculin, and negatively to both controls. One hundred and ninety-five children gave negative reactions to all antigens at both times.

Prison. Sixty-five prisoners (27.8 per cent) gave positive reactions to trichinella antigen at some time (Table I). Fifty-two were immediate

TABLE I
Summary of trichinella and tuberculin skin tests

Institution	Number tested	Trichinella								Tuberculin						
		30 minutes			24 hours			Total		30 minutes			24 hours			
		Positive	Test and control positive	Negative	Positive	Test and control positive	Negative	Positive at either time	Percentage positive	Positive	Test and control positive	Negative	Positive	Percentage positive	Test and control positive	Negative
Orphanage	285	51	7	227	9	0	276	57*	20.0	60	164	61	40	14.0	0	245
Prison (male)	183	43	12	128	13	1	169	53*	29.0	25	72	86	14	7.6	1	168
Prison (female)	58	9	1	48	3	0	55	12	20.7	9	19	30	3	5.2	0	55
Total	526	103	20	403	25	1	500	122	23.2	94	255	177	57	10.8	1	468

* Three patients had positive reactions at both 30 min. and 24 hrs.

reactions, and 16 were delayed. Three were positive at both 30 minutes and 24 hours. Thirteen prisoners gave positive reactions to both the test and control solution at 30 minutes. Seventeen prisoners (7.1 per cent) had delayed reactions to tuberculin. Two prisoners reacted to both tuberculin and trichinella antigen, and did not react to the control solutions. One prisoner gave a positive reaction to both tuberculin and the tuberculin control solution at 24 hours. One hundred and sixty-three prisoners reacted negatively to all antigens at both times.

TABLE II

Comparison of tuberculin and trichinella skin tests

Institution	Trichinella positive (either time)	Tuberculin (24 hrs.) 0	1+	2+	3+	4+
Orphanage	0	195	23	9	1	0
	1+	21	3	1	0	0
	2+	14	1	0	0	0
	3+	9	1	0	1	0
	4+	6	0	0	0	0
Prison (male)	0	120	9	1	0	0
	1+	19	0	0	1	0
	2+	16	2	0	1	0
	3+	11	0	0	0	0
	4+	3	0	0	0	0
Prison (female)	0	43	2	1	0	0
	1+	7	0	0	0	0
	2+	2	0	0	0	0
	3+	3	0	0	0	0
	4+	0	0	0	0	0

Since the interpretation of immediate and delayed reactions to trichinella antigen is still in doubt, positive reactions at either period were compared with late tuberculin reactions (Table II). No correlation between positive reactions to tuberculin and trichinella antigen was found.

The data were tabulated according to the extent of the trichinella reaction and the duration of stay in each institution (Table III). The duration of stay in an institution did not appear to affect the extent of the trichinella reaction. When the percentage of positive trichinella reactions was tabulated according to years of institutionalization, the incidence was found to be highest in the orphanage at the second and fifth years, with a secondary peak at the ninth year. In the prison, the peak incidence was reached at the sixth year.

TABLE III

Comparison of extent of reaction to trichinella skin tests with duration of stay in an institution

Years	Orphanage 0	1+	2+	3+	4+	Number	Positive	Per cent	Prison (males) 0	1+	2+	3+	4+	Number	Positive	Per cent	Prison (females) 0	1+	2+	3+	4+	Number	Positive	Per cent	Total Tested	Positive	Per cent
1	10	0	1	1	0	12	2	16.6	25	3	4	3	0	35	10	28.6	7	1	0	0	0	8	1	12.5	55	13	23.6
2	34	6	1	3	1	45	11	24.4	12	3	1	0	1	17	5	29.4	17	3	0	1	0	21	4	19.0	83	20	24.1
3	41	4	3	2	1	51	10	20.0	21	3	3	1	0	28	7	25.0	6	0	1	0	0	7	1	14.6	86	18	20.9
4	22	2	1	2	1	28	6	21.6	9	1	2	2	0	14	5	35.7	3	1	0	1	0	5	2	40.0	47	13	27.7
5	36	4	5	0	2	47	11	23.4	12	1	3	2	1	19	7	36.8	6	0	0	0	0	6	0	0.0	72	18	25.0
6	20	2	2	2	0	26	6	23.0	8	2	3	0	1	14	6	42.9	1	1	0	1	0	3	2	66.6	43	14	31.8
7	12	1	0	1	0	14	2	14.3	13	5	1	1	0	20	7	35.0	2	1	0	0	0	3	1	33.3	37	10	27.0
8	7	2	0	0	0	9	2	22.2	9	1	0	1	0	11	2	18.3	0	0	0	0	0	0	0	0.0	20	4	20.0
9	11	2	2	0	0	15	4	26.6	6	1	0	0	0	7	1	14.6	1	0	1	0	0	2	1	50.0	24	6	25.0
10	17	1	0	1	1	20	3	15.0	9	1	1	0	0	11	2	18.3	1	0	0	0	0	1	0	0.0	32	5	15.6
11 to 15	17	3	0	0	0	20	3	15.0	6	2	1	1	0	10	4	40.0	2	0	0	0	0	2	0	0.0	32	7	21.8
16 to 20	1	0	0	0	0	1	0	0.0	0	0	0	0	0	0	0	0.0	0	0	0	0	0	0	0	0.0	1	0	0.0
Total	228	27	15	12	6	288	60*	20.8	130	23	19	11	3	186	56**	30.0	46	7	2	3	0	58	12	20.7	532	128	24.1

* Three orphans had both immediate and delayed positive tests.

** Three prisoners had both immediate and delayed positive tests.

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TABLE IV

Comparison of serologic reactions for trichinosis with skin tests

Institution	Positive trichinella skin test							Positive tuberculin skin test							Negative trichinella and tuberculin skin test (control)					
	Total positive at either time	Flocculation			Precipitin			Positive at 24 hours	Flocculation			Precipitin			Flocculation			Precipitin		
		Tested	Positive	Negative	Tested	Positive	Negative		Tested	Positive	Negative	Tested	Positive	Negative	Tested	Positive	Negative	Tested	Positive	Negative
Orphanage	57	38	0	38	0			40	20	0	20	0			20	0	20	0		
Prison (male)	53	44	4(±)*	40	20	0	20*	14	5	0	5	5	0	5	31	0	31	18	0	18
Prison (female)	12	9	1(±)*	8	4	0	4**	3	0			0			20	2(±)* 2(3+)**	16	14	0	14*
Total	122	91	5	86	24	0	24	57	25	0	25	5	0	5	71	4	67	32	0	32

* Sera with doubtful (±) flocculation gave negative precipitin test.
** Sera with 3+ flocculation gave negative precipitin test.

Serologic reactions

Orphanage. Flocculation tests for trichinosis were performed on serum from 38 children with positive trichinella skin tests, from 20 children with positive tuberculin and negative trichinella skin tests, and from 20 who reacted negatively to all antigens. All flocculation tests were negative (Table IV). No precipitin tests were done on the children.

Prison. Flocculation tests were performed on serum from 53 prisoners with positive reactions to trichinella antigen, from 5 with positive delayed tuberculin reactions, and from 51 who reacted negatively to all antigens (Table IV). Five sera from the group with positive trichinella skin tests, and 2 from the group negative to both antigens, gave doubtful flocculation reactions; 2 of the latter group gave strongly positive flocculation reactions. Precipitin tests were performed on serum from 20 prisoners with positive trichinella reactions, from 5 with positive tuberculin reactions, and from 18 who were negative to all antigens; none were positive. Serum had been collected from the other prisoners after a meal and was too cloudy for performance of accurate precipitin tests. Precipitin tests on serum from rabbits known to be infected with trichinae were strongly positive; these tests served as a control on the technique and the potency of the antigen.

DISCUSSION

No correlation has been found between the tuberculin and trichinella skin reactions; this clinical observation agrees with the results of experimental investigations in rabbits (3). No biologic cross reaction detectable by skin test appears to exist; the increased incidence of positive trichinella skin tests in tuberculous patients is still unexplained.

Whether the apparent increase in the incidence of positive trichinella skin tests among patients confined to any institution is connected with subclinical institutional epidemics, cannot be categorically stated. The fact that the flocculating antibodies and precipitins appear to bear no relation to positive skin reactions would argue against subclinical institutional epidemics. However, precipitins are known to persist for 2½ years after proven infection in human beings (5). The length of time for which flocculating antibodies persist in human beings is not known, but in swine the test remains positive for 10 months (4). It is possible that different antigens are involved in the production of the skin test and the serum reactions (6).

The increase in the incidence of positive skin reactions previously noted after 2 years' residence in an institution was confirmed at the orphanage; at the prison the peak was later, but in general the relation of positive skin tests to length of institutionalization followed the data previously reported (1). The explanation for this relationship is still obscure.

The wide variation in the incidence of positive reactions obtained in the 9 institutions which have now been studied cannot yet be explained.

While a positive tuberculin skin reaction does

not indicate the presence of active tuberculosis, it does indicate that the individual has been infected at some time and is allergic to some fraction of the organism. It would be reasonable to expect some degree of correlation between the skin reactions to tuberculin and trichinella antigen if one disease affects the severity of the other, as was suggested by the experiments in guinea pigs previously reported (7). Perhaps active tuberculosis is necessary for the infection with trichinae to be enhanced; the present study cannot answer this question.

That tuberculosis may affect the course of trichinosis was shown also by studies of the eosinophil response of guinea pigs (8). The injection of live virulent *M. tuberculosis* depressed the number of circulating eosinophils in trichinous animals. Since a rapid rise in eosinophils is the most frequent and easily detected early laboratory evidence of uncomplicated trichinosis, the absence of eosinophilia in a tuberculous patient with a positive trichinella skin test could cause confusion in diagnosis.

It is known that the electrical resistance of the skin of disturbed mental patients may vary markedly from that of the same patient after recovery (9). Previous studies with trichinella antigen in one mental hospital suggested that the reactivity to biologic products may be altered in the same way (1). Though none of the prisoners or orphans gave a history of active mental disease, it is true that all prisoners have committed an offense and may justly be suspected of having a psychiatric disturbance. Many orphans may likewise have some mild psychiatric manifestations. None of the individuals studied was acutely disturbed, however, and hence would not be expected to have an altered skin reaction.

SUMMARY

1. No correlation between the trichinella skin test and the tuberculin reaction was found in 526 individuals confined in an orphanage or a prison. The incidence of positive trichinella skin tests in 241 prisoners was 27.8 per cent; the incidence in 285 orphans was 20.0 per cent.

2. Positive serologic reactions to the flocculation or precipitin tests were not found in persons with positive trichinella skin reactions.

3. The duration of stay in an institution is apparently a factor in determining the incidence of positive trichinella skin tests. The explanation for this finding is obscure.

BIBLIOGRAPHY

1. Harrell, G. T., and Horne, S. F., Trichinella skin tests in tuberculosis sanatoriums, hospitals for mental diseases, and general hospitals. A comparison of results in tuberculous and non-tuberculous patients. Am. J. Trop. Med., 1945, 25, 51.
2. Avera, John W., Yow, E. M., Harrell, G. T., and Fowler, E. B., An attempt by feeding to induce in animals reactivity to *Trichinella spiralis* in the absence of infection. Am. J. Trop. Med., 1946, 26, 125.
3. Harrell, G. T., and Helsabeck, N. J., The effect on the trichinella skin test of simultaneous infection with bovine tuberculosis in trichinous rabbits. J. Clin. Invest., 1947, 26, 69.
4. Suessenguth, H., and Kline, B. S., A simple rapid flocculation slide test for trichinosis in man and in swine. Am. J. Clin. Path., 1944, 14, 471.
5. Gould, S. E., Trichinosis. Charles C. Thomas, Springfield, Illinois, 1945, p. 143.
6. Aikawa, J. K., Harrell, G. T., and Helsabeck, N. J., The effect of peptic and tryptic digestion on the antigenicity of *Trichinella spiralis*. J. Clin. Invest., 1947, 26, 73.
7. Davis, O. T., Harrell, G. T., and King, E. S., The effect of simultaneous tuberculous infection on experimental trichinella infestations in guinea pigs. Am. J. M. Sc., 1945, 209, 758.
8. Spink, W. W., Effects of vaccines and bacterial and parasitic infections on eosinophilia in trichinous animals. Arch. Int. Med., 1934, 54, 805.
9. Barnell, A., Correlations between epidermal impedance and the clinical course in certain psychoses. Proc. Soc. Exper. Biol. and Med., 1939, 42, 839.

THE EFFECT ON THE TRICHINELLA SKIN TEST OF SIMULTANEOUS INFECTION WITH BOVINE TUBERCULOSIS IN TRICHINOUS RABBITS [1]

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The possibility that infection with tuberculosis alters an individual's reactivity to trichinella antigen was suggested by the results of a survey on the incidence of trichinosis. It was noted that a higher percentage of positive reactions to trichinella antigen (18.3 per cent) was obtained in 674 patients with active tuberculosis in 3 sanatoria, than in 335 patients without tuberculosis (7.2 per cent) in 2 general hospitals (1). The explanation for this phenomenon has not been found by investigations on the possible effects of institutionalization and sensitization by the ingestion of nonviable trichinae (2, 3). The possibility of a biologic cross reaction, detectable by skin test, between *Mycobacterium tuberculosis* and *Trichinella spiralis* was investigated in guinea pigs infected simultaneously with tuberculosis and trichinosis (4). Because of the poor response of the guinea pig to trichinella skin test antigen, the possibility of such a cross reaction was neither confirmed nor denied, but it was found that guinea pigs infected simultaneously with both organisms showed heavier infections with *T. spiralis* than did similar animals suffering from trichinosis alone. The converse of this statement was not true, since the number of acid-fast organisms found on smears of the tuberculous lesions in trichinous guinea pigs was no greater than that observed in similar animals infected with *Mycobacterium tuberculosis* alone. It was postulated that an increased density of infection with trichinae in patients with active tuberculosis might explain the higher incidence of positive trichinella skin tests in such patients.

The present experiments were undertaken in an attempt to discover whether a true biologic cross reaction detectable by skin test exists between *Mycobacterium tuberculosis* and *Trichinella spiralis*. Since rabbits have been found to give better skin reactions to trichinella antigen than guinea pigs, this animal was selected for these experiments. This choice necessitated the use of a bovine strain of tubercle bacilli (2). Dogs have been found to give excellent skin reactions to trichinella antigen, but in preliminary experiments it was difficult to establish a uniform bovine tuberculous infection in them.

MATERIALS

Sixty rabbits of medium size were divided into 6 equal groups. The rabbits were between 4 and 9 weeks old, and weighed 2¼ to 7½ pounds. They were fed a stock diet (Kasco Complete Rabbit Ration, Kasco Food Co., Cincinnati, Ohio) supplemented with green vegetables 3 times weekly. Each animal was kept in a separate cage, and the groups infected with tubercle bacilli were kept in a separate room from those infected with trichinae alone.

The strain of *Trichinella spiralis* used was obtained from the National Institute of Health and was carried in albino rats. The culture of *Mycobacterium tuberculosis* (bovine, Ravenel strain) was obtained from Dr. Florence Seibert of the Henry Phipps Institute of the University of Pennsylvania, and was carried on plain glycerol bouillon agar medium titrated to pH 7.4. This highly virulent strain has been used by Opie and Freund (5). Intravenous injections of 0.5 ml. of a suspension of 1 loopful of culture in 5 ml. of saline solution resulted in death of a rabbit within 24 days.

Part of the dried trichinella larvae used was furnished by Parke, Davis and Co., Detroit, Michigan; the remainder was recovered from infected rabbits in our own laboratory. For skin testing, a 1:200 weight/volume emulsion in normal saline was made with dried powdered larvae, and was allowed to stand for 4 hours at room temperature, and overnight in the refrigerator at 12° C. The supernatant saline was then removed, placed in sterile rubber-stoppered vials, heated in a water bath at 60° C. for 1 hour daily on 3 successive days, and tested for sterility (2).

Tuberculin prepared from bovine strains of tubercle bacilli was furnished by Sharpe and Dohme, Glenolden, Pennsylvania. Purified protein derivative (PPD) of human strains of tubercle bacilli, furnished by Dr. Seibert, was also used; rabbits infected with bovine strains of tubercle bacilli react to this material (6).

[1] This study was aided by a grant from the John and Mary R. Markle Foundation.

METHODS

Experiment 1: Skin tests

Groups B and C were fed approximately 2 grams of meat which had been obtained from a rat heavily infected with trichinae 26 days previously. On the following day, groups A and C were injected in the groin with 0.1 ml. of a suspension of tubercle bacilli.[2] Thirty-nine days after the injections, the abdomen of the animals was clipped, and skin tests were done with 0.1 ml. of a 1:100 dilution of tuberculin in distilled water, with 0.1 ml. of physiological saline solution containing 0.005 mgm. PPD, with 0.02 ml. of 1:200 trichinella antigen, and with 0.1 ml. of saline solution as a control. The readings were made at 20 minutes, 24 hours, and 48 hours.

Experiment 2: Intensity of trichinella infection

Forty-five days after the injections, groups A and C were killed with a blow on the head; the rabbits in group B were killed 54 to 64 days after infection with trichinae. Three-tenths to 0.35 gram of muscle was removed from the right anterolateral aspect of the diaphragm, and from each of the right front and hind legs; 0.25 to 0.3 gram of intercostal muscle was removed. Because the degree of infection previously found in guinea pigs had varied so greatly between the group infected with both trichinosis and tuberculosis and the group infected with trichinosis alone, the samples were not accurately weighed, but were estimated (4). The tissues were examined in a muscle press using the 10 × low power objective and 10 × ocular lenses in the microscope. The absolute number of trichinae observed in each sample was recorded. Since the muscle press preparations on 2 animals (numbers 22 and 23 from group C) were negative, approximately 5 grams of muscle obtained from various sites in these animals was digested with 0.7 per cent pepsin and 1 per cent hydrochloric acid (7).

Groups E and F were fed 2 grams of trichinous rat meat, and the following day groups D and F were injected with 0.05 ml. of a 5 ml. suspension of tubercle bacilli prepared by the method described above. Two animals in group E (numbers 13 and 16) were fed 7 days later than the remainder of the group, and their meat was prepared from a different rat. The animals were killed 70 to 86 days after infection, and the muscles examined in the manner described. Smears were made from tuberculous lesions in the lung, the liver, the regional lymph nodes, and at the site of injection in the groin. Smears were stained by the Ziehl-Neelsen technique, and the data recorded by the Gaffky scale.

[2] The suspension was made from 1 medium-sized colony of a 6-weeks culture ground with 1 drop of sterile physiological saline in a small mortar. The suspension was subsequently diluted with saline to make the volume 10 ml. and the large clumps were centrifuged out; the supernatant fluid showed 10 to 20 organisms per field with an oil-immersion lens.

RESULTS

One animal in group C died before the skin tests were done, and 2 animals in group D died of intercurrent disease before the experiments were completed. None of these animals is included in the results.

In 2 animals in group C (numbers 22 and 23) no trichinae were found in any muscle press preparation, and digestion of muscle revealed no live trichinae. One animal from group B, 1 from group E, and 2 from group F had very light infections, 1 to 3 trichinae being seen in muscle from 1 site only.

Each animal in groups A, C, D, and F presented gross evidence of tuberculosis at autopsy. In 1 animal from group D, and 1 from group F, smears of tuberculous lesions were negative; in all others acid-fast bacilli were found.

Experiment 1: Skin tests

No essential difference in reaction to the 2 trichinella antigens was noted (Table I). Twelve

TABLE 1

Skin tests in trichinous and tuberculous rabbits

Group	Number animals	Tuberculin	Purified protein derivative	Trichinella antigen no. 1	Trichinella antigen no. 2	Saline control
A (tuberculosis)	10	3.2	3.5	0.1	0	0
B (trichinosis)	10	0	0.1	1.6	2.0	0
C (tuberculosis and trichinosis)	9	3.6	4.1	2.1	1.1	0

The data are the arithmetic means of the scores of skin tests on a scale of 0 to 4 plus; doubtful (±) reactions are calculated as negative (0).

Trichinella antigen no. 1 was prepared in our own laboratory; the larvae of antigen no. 2 were furnished by Parke, Davis and Co.

animals gave immediate positive skin reactions to trichinella antigen at 20 minutes; 11 animals gave 1 plus or greater reactions at 24 or 48 hours. Five animals, proved by muscle press preparations to be infected, gave negative skin reactions at all 3 periods to both antigens. One infected animal which failed to react at 20 minutes, gave a positive reaction at 24 hours, while 3 animals positive at 20 minutes failed to give reactions at 24 and 48 hours. One false positive reaction (1 plus) to a single trichinella antigen was encoun-

tered in a tuberculous animal which had not been fed trichinae.

No difference in reactions to bovine tuberculin and human PPD was noted in the infected animals. None of the tuberculous animals gave immediate reactions to either antigen, and only 1 animal gave a 1 plus reaction; all of the other animals gave 3 or 4 plus tests. No false positive reaction to tuberculin or PPD was encountered in non-tuberculous animals.

Experiment 2: Intensity of trichinella infection

No essential difference in intensity of trichinous infections was found between groups B and C, and between groups E and F (Table II). This

TABLE II

Density of trichinella infection of muscle

Group	Number animals	Diaphragm	Intercostal	Front leg	Hind leg	All sites
B (trichinosis)	10	65.3	14.2	28.5	17.6	31.4
C (trichinosis and tuberculosis)	9	42.9	4.2	12.1	5.9	16.3
E (trichinosis)	10	174.4	68.8	76.5	60.8	95.1
*	(8)	(125.0)	(60.1)	(45.2)	(24.6)	(63.7)
F (trichinosis and tuberculosis)	10	114.0	43.7	42.2	15.7	53.9

The data are the arithmetic means of the counts of larvae as seen in the muscle press. Groups B and C were fed on the same day, and groups E and F on another occasion.

* The data for group E have also been calculated omitting rabbits number 13 and 16 which were fed from a more heavily infected rat 7 days later than the remainder of the group. These data are recorded in parentheses for more accurate comparison with group F.

was true for the counts of trichinae in muscle from any single site, as well as for all sites calculated together.

No difference in intensity of tuberculous infection was noted between groups D and F either in the number and extent of gross lesions, or in the number of organisms seen on smears of the lesions.

DISCUSSION

No biologic cross reaction between *Mycobacterium tuberculosis* and *Trichinella spiralis* was detected by skin tests in rabbits. Judged by the extent of positive reactions to the concentrations of antigens used, tuberculin and PPD are more specific for tuberculosis than is trichinella antigen for trichinosis. The failure of 4 animals with proved trichinosis to react to trichinella skin tests could not be accounted for either by overwhelming or by very light infections. Only 1 of these animals had a very light infection; the other 3 had infections which were average in density. A single animal gave an immediate false positive skin reaction to one trichinella antigen, though no trichinae had been fed to it.

The differences in reaction to trichinella antigen at 20 minutes, 24 hours and 48 hours are of interest in the light of experiments recently performed on dogs, using trichinella antigen digested with pepsin or trypsin, and presumably containing no reaginic protein (8). It is impossible at this time to say whether the immediate reactions at 20 minutes in rabbits are due to carbohydrate and the late reactions at 24 and 48 hours to protein, as the experiments in dogs might suggest.

Further experiments have been carried out in orphanage and prison groups, in order to compare the tuberculin and trichinella skin reactions in non-tuberculous, non-psychiatric institutionalized persons. These clinical experiments, which are being reported separately (3), confirm the animal experiments reported here; no biologic cross reaction seems to exist between the 2 infectious agents.

The experiments in rabbits infected simultaneously with bovine *Mycobacterium tuberculosis* and *Trichinella spiralis* reported here do not confirm the previously reported experiment in guinea pigs infected simultaneously with human *Mycobacterium tuberculosis* and *Trichinella spiralis* (4). The density of trichinous infection in rabbits simultaneously infected with trichinosis and tuberculosis was not found to be greater than that in rabbits infected with trichinosis alone.

SUMMARY

1. No biologic cross reaction was detected by skin tests in rabbits infected simultaneously with bovine *Mycobacterium tuberculosis* and *Trichinella spiralis*.

2. Simultaneous infection with bovine tuberculosis did not increase the intensity of trichinous infections in rabbits.

BIBLIOGRAPHY

1. Harrell, G. T., and Horne, S. F., Trichinella skin tests in tuberculosis sanatoriums, hospitals for mental diseases, and general hospitals. Am. J. Trop. Med., 1945, 25, 51.
2. Avera, J. A., Yow, E. M., Harrell, G. T., and Fowler, E. B., An attempt by feeding to induce in animals reactivity to *Trichinella spiralis* in the absence of infection. Am. J. Trop. Med., 1946, 26, 125.
3. Harrell, G. T., Horne, S. F., Aikawa, J. K., and Helsabeck, N. J., Trichinella skin tests in an orphanage and prison: comparison with the flocculation test and with the tuberculin reaction. J. Clin. Invest., 1947, 26, 64.
4. Davis, O. T., Harrell, G. T., and King, E. S., The effect of simultaneous tuberculous infection on experimental trichinella infestations in guinea pigs. Am. J. M. Sc., 1945, 209, 758.
5. Opie, E. L., and Freund, J., Experimental study of protective inoculation with heat killed tubercle bacilli. J. Exper. Med., 1937, 66, 761.
6. Personal communication.
7. Bozicevich, J., Studies on trichinosis. XII. The preparation and use of an improved trichina antigen. Pub. Health Rep., 1938, 53, 2130.
8. Aikawa, J. K., Harrell, G. T., and Helsabeck, N. J., The effect of peptic and tryptic digestion on the antigenicity of *Trichinella spiralis*. J. Clin. Invest., 1947, 26, 73.

THE EFFECT OF PEPTIC AND TRYPTIC DIGESTION ON THE ANTIGENICITY OF *TRICHINELLA SPIRALIS*[1]

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In the course of a previous investigation, positive trichinella skin tests which could not be accounted for by a history of known infection were encountered (1). An attempt was made to explain these findings by inducing reactivity in non-infected animals through repeated feedings of antigen (2). Killed larvae of *Trichinella spiralis,* presumably containing denatured (cooked) or undenatured (frozen) antigen, were fed to guinea pigs and rabbits. Attempts to demonstrate reactivity in these animals by skin tests and flocculation tests on the blood serum were unsuccessful. Because some investigators have found that digestion may alter the reactivity of pollens, it was thought that the negative results might have been due to digestion of the trichinella antigen (3, 4). The present study was undertaken to determine whether or not peptic or tryptic digestion would deactivate the antigen of killed trichinae.

MATERIALS

Animals

Fifteen young dogs weighing 5 to 15 kgm. were de-wormed and divided into 3 groups of 5 each (groups A, B, and C). A fourth group of 15 dogs (group D) which had not been de-wormed was used as a control. All animals were fed a stock diet (Hunt Club Dog Food, Maritime Milling Co., Inc., Buffalo, N. Y.)

Trichinella larvae

The strain of *Trichinella spiralis* which we employed was obtained from the National Institute of Health and was carried in albino rats. Rabbits were given 1 feeding of trichinae-infected rat meat, and 8 weeks later were killed, skinned, and eviscerated. The muscle was removed, ground, and digested at 37° C. for 6 hours in a solution of 0.7 per cent pepsin and 1.0 per cent hydrochloric acid (5). The larvae were washed repeatedly in normal saline until the solution was biuret-negative, and were then frozen at −78° C. for 72 hours in carbon-dioxide ice, dried in a desiccator, and powdered.

METHODS

Preparation of the alkaline-trypsin digested antigen

Fifty mgm. of powdered trichinae were incubated for 48 hours with 5 ml. of 5 per cent trypsin (Armour and Co., Chicago, Ill.) in a 0.3 per cent sodium carbonate solution at 37° C. under anaerobic conditions. The anaerobic environment was chosen because Rockwell has suggested that oxidation may destroy antigenicity (6). As soon as the trichinae were added, a 22 × 200 mm. test tube was inverted over the 10 × 100 mm. unstoppered tube containing the digestion mixture, and a cotton plug was pushed into the larger tube to hold the smaller tube in place. The mouth of the larger tube was then placed in a beaker containing a mixture of pyrogallic acid powder and sodium hydroxide pellets. About 10 to 15 ml. of water were added to the reagents, and melted paraffin was poured on its surface. As the oxygen was utilized, the fluid column rose, effectively sealing the tube.

After digestion, the mixture was centrifuged for 30 minutes at approximately 3,000 RPM. The supernatant fluid was then removed with a pipet and neutralized to litmus with 5 per cent hydrochloric acid. Sufficient phenol was added as a preservative to make a final concentration of 0.04 per cent. A biuret test on the sediment was negative; the sediment was discarded.

A test for sterility on the solution of digested antigen revealed Gram-positive spore-bearing rods which were not further identified. Because of the contamination, the antigen was frozen for 48 hours in carbon-dioxide ice. A subsequent sterility test was negative. The Gram-positive bacilli were found to be a contaminant in the trypsin powder.

Preparation of the acid-pepsin digested antigen

Fifty mgm. of powdered trichinae were incubated for 48 hours with 5 ml. of 0.7 per cent pepsin (Merck, N.F. Granular) in 1 per cent hydrochloric acid at 37° C. under anaerobic conditions, by the method described above. The supernatant fluid, after centrifugation, was neutralized to litmus with 5 per cent sodium hydroxide. The sediment left after centrifugation was found to be very slightly biuret-positive; it was then discarded.

A sterility test on the digested antigen was negative, but in order to maintain conditions identical with the alkaline-trypsin digestion, the antigen was frozen for 48 hours in carbon-dioxide ice.

Preparation of the control antigen

Twenty-five mgm. of powdered trichinae were incubated anaerobically for 48 hours in 2.5 ml. of sterile 0.8

[1] This study was aided by a grant from the John and Mary R. Markle Foundation.

per cent sodium chloride solution. The mixture was then centrifuged and frozen exactly as in the preparation of the digested antigens. A biuret test on the sediment was strongly positive. A sterility test on the incubated antigen was negative.

Injections

Group A received the alkaline-trypsin digested antigen, group B the acid-pepsin digested antigen, and group C the undigested control antigen. Each animal was given an initial subcutaneous injection of 0.1 ml. of its respective antigen in the left pectoral region. One dog from each group (dogs 5, 10, and 14) was also given 0.1 ml. of its respective antigen intravenously on the same day, without reaction. Beginning 3 days later, all animals were given 3 successive daily intravenous injections of 0.1 ml. of their respective antigen, a total of 0.4 ml. of antigen to each dog, except dogs 5, 10, and 14, which received 0.5 ml.

Skin tests

In order to avoid the possibility of sensitization, no skin tests were performed before the injections were given. Nineteen days after the last intravenous injection, the abdomen of each animal in groups A, B, and C was clipped, and a skin test was done on each animal with a 1:100 dilution of antigen, prepared from desiccated and powdered trichinae recovered from the same rabbit that supplied the trichinae for the injected antigen. At the same time, each animal was given a skin test with a saline control. Subsequent skin tests with a 1:200 dilution of antigen from another rabbit were done at intervals of approximately 1 week through the sixth week after the last intravenous injection. All antigens used were found to give 4 plus reactions in a dog known to be infected with trichinae.

The reactions were read at 20 minutes. A 1 cm. wheal with surrounding erythema was considered to be a 1 plus reaction, if the skin test with a saline control produced a wheal less than 8 mm. in diameter. Reactions were graded progressively from 1 plus to 4 plus.

As a further control against non-specific or group skin reactions, 15 additional dogs (group D) which had not been de-wormed, or given any injections of antigen, were given skin tests with a 1:200 dilution of antigen.

Flocculation tests

Preliminary flocculation tests were done on the serum obtained from each dog in groups A, B, and C 9 days before the injections of antigen were begun. Eleven days after completion of the intravenous injections, 5 ml. of blood were obtained from the antecubital vein of each dog. The serum was separated and inactivated at 56° C. for 30 minutes. Tests were performed according to the technique of Suessenguth and Kline (7), except that the rotation was continued for at least 10 minutes. Judging by the negative control, this longer time interval did not give false positive results. Minimal but definite flocculation which could be seen only under the microscope was considered a doubtful reaction (±). Flocculation tests were repeated at intervals of approximately 1 week through the sixth week after the last intravenous injection.

RESULTS

Skin tests

In 13 of the 15 dogs in groups A, B, and C, skin tests were found to be positive on the nineteenth day after the initial injection, and continued to be positive for the duration of the experiment (Table I). One animal (dog 5) in group A (alkaline-trypsin digested antigen) failed to develop a definitely positive skin test during the 6-week period of observation. One animal (dog 9) in group B (acid-pepsin digested antigen) failed to react at 19 days, but subsequently developed a positive skin test. No difference in the extent of reactions was noted among the 3 test groups.

TABLE I

Skin tests

	Dog	Days following initial injection			
		19	24	30	41
Group A	1	++	+	+	++++
Alkaline-	2	+++	++++	++++	++
trypsin	3	+	++++	++++	++++
digested	4	++	++	+	++++
antigen	5	0	0	±	±
Group B	6	+	+++	++	++
Acid-pepsin	7	+	++	++++	++++
digested	8	+	0	±	++
antigen	9	0	+	+	+
	10	+	±	+	++++
Group C	11	+++	++	++	++
Saline	12	+	+++	+++	++
antigen	13	+	+++	++	++++
control	14	+	++	++	++++
	15	++	++++	++++	++++

Of the 15 dogs in group D (the control group of dogs which had not been de-wormed and had not received injections of antigen), 5 gave negative reactions, 5 gave doubtful (±) reactions, 4 gave 1 plus reactions, and 1 gave a 2 plus reaction at 20 minutes. Two gave 1 plus reactions at 24 hours.

Flocculation tests

Preliminary flocculation tests on the sera of all dogs in groups A, B, and C were negative (Table II). On the twenty-first day, tests on all these dogs gave at least doubtful reactions. No striking difference in the flocculation tests was.noted among

TABLE II

Flocculation tests on serum

	Dog	Preliminary	Days following initial injection: 16	21	35	41
Group A Alkaline-trypsin digested antigen	1	0	0	++	±	+
	2	0	0	+	±	+
	3	0	0	±	±	+
	4	0	0	±	0	±
	5	0	0	+	±	+
Group B Acid-pepsin digested antigen	6	0	0	+	++	++
	7	0	0	++	0	0
	8	0	0	+	±	0
	9	0	0	++	+	+
	10	0	0	+++	+	+
Group C Saline control antigen	11	0	0	+	±	±
	12	0	0	±	±	0
	13	0	0	+	±	±
	14	0	0	++++	±	±
	15	0	0	+++	+	±

the 3 test groups. In general, the skin tests gave more strongly positive reactions than did the flocculation tests.

DISCUSSION

The effect of digestion on antigenicity

Acid-pepsin and alkaline-trypsin digestion of trichinella antigen did not destroy its reactivity for dogs, as measured by skin tests and flocculation tests on serum. Our results suggest that the antigenicity of trichinae does not reside solely in the protein fraction. Jadassohn found that peptic digestion of ascaris antigen did not alter its reactivity for skin tests (8). The results of our studies on another helminth parasite agree with his findings.

The reports in the literature concerning the effect of digestion on the antigenicity of grass pollens are conflicting. Coca and Grove were unable to find any diminution, as measured by skin tests, in the activity of ragweed and timothy pollen extracts after digestion with trypsin (10). Black obtained similar quantitative reactions with ragweed pollen extracts, as measured by endermic and intranasal tests, before and after tryptic digestion (11). He concluded that the substance responsible for pollen sensitization is not a protein, that it survives passage through the alimentary tract, and that it can be recovered from blood, urine, and feces (12). Thiberge, however, showed a reduction in the skin-test activity of ragweed pollen extracts after artificial digestion with pepsin and hydrochloric acid (3). Pancreatin digestion was said to intensify the reactivity of these extracts, but details of the technique used were not given (9). Harsh and Huber found that peptic or tryptic digestion of giant ragweed pollen caused a marked loss of activity of the antigen, as measured by scratch and intracutaneous tests (4). A small but constant amount of activity which was unaffected by proteolytic digestion remained.

Thiberge demonstrated by a passive transfer test (Prausnitz-Kustner phenomenon) that ragweed antigen was absorbed in an active form from the intestinal tract after being administered in enteric-coated pills. The preponderance of evidence indicates that the proteolytic enzymes of the gastro-intestinal tract destroy little of the active substance in pollen antigen. Little is known concerning the effect of carbohydrate-splitting enzymes on the antigen.

The experiments reported here do not explain, on the basis of digestion of antigen, the failure to induce reactivity to trichinella antigen in animals by repeated feeding of killed trichinae (2). The possibility that ingestion of killed trichinae by human beings may cause sensitization without actual infection still remains (1).

The antigenicity of trichinae fractions

Melcher, by chemical methods which did not employ enzymatic digestion, separated a polysaccharide and 2 protein fractions from trichinae (13). In infected rabbits, the protein fractions gave positive skin reactions at 24 hours, while the polysaccharide gave negative skin tests at 24 hours, but gave strongly positive precipitin reactions. The polysaccharide also induced precipitin formation when injected in non-infected animals, and the serologic studies suggested that the carbohydrate was associated with the acid-soluble protein fraction. Our experiments with dogs would suggest that the positive skin reactions at 20 minutes to antigen freed of protein by digestion might be due to a carbohydrate which need not be the same as that studied by Melcher. The experiments were done on a different species of animal, and the tests were read at a different time, but in human beings with pneumococcal infections the reaction in the skin to injected polysaccharide occurs at 20 minutes.

Bachman reported that the active antigenic fraction of trichinae is a glucoprotein, a substance chemically similar to mucin and containing carbohydrate in the molecule. He did not attempt to split the compound and study the protein and carbohydrate fractions separately (14). Cross-reactions between trichinae and other parasites, which occur at low dilutions of antigen (1:100), tend to disappear at high dilutions (1:10,000) (15). It is not known whether the cross-reactions are associated with a polysaccharide or with a protein fraction.

Proteolytic digestion of the trichinella antigen employed for skin tests may be found to reduce the incidence of non-specific false positive reactions commonly encountered with the protein-containing antigen now used.

The relative sensitivity of skin test and serologic methods

The fact that the skin tests gave more strongly positive reactions than the flocculation tests may indicate that the skin gives a greater quantitative response, and hence that the skin test is more sensitive. However, dogs known to be infected give 4 plus reactions to both skin and flocculation tests. The difference in intensity of reaction between the flocculation tests and the skin tests might be explained on the basis of 2 antigens, one elaborated continuously by the living larvae and resulting in flocculating antibodies; the other, an incomplete antigen incapable by itself of producing flocculating antibodies, and requiring the presence of another antigen not found in extracted whole killed trichinae, but present in living trichinae. Recent experiments by Roth suggest that precipitation of low titer immune serum around *living trichinae* is a more sensitive test than precipitation produced by antigen from dead parasites (16).

SUMMARY

1. Acid-pepsin and alkaline-trypsin digestion of *Trichinella spiralis* antigen did not destroy its antigenicity for dogs, as measured by skin tests and flocculation tests on serum.

2. The more marked reactivity of the skin test as compared with the flocculation test suggests that the same antigen may not be responsible for both reactions.

3. The failure of proteolytic digestion to destroy the antigens suggests that the antigenic fraction of *Trichinella spiralis* is not solely a protein.

BIBLIOGRAPHY

1. Harrell, G. T., and Horne, S. F., Trichinella skin tests in tuberculosis sanatoriums, hospitals for mental diseases, and general hospitals. Am. J. Trop. Med., 1945, 25, 51.
2. Avera, J., Yow, E., Harrell, G. T., and Fowler, E. B., An attempt by feeding to induce in animals reactivity to *Trichinella spiralis* in the absence of infection. Am. J. Trop. Med., 1946, 26, 125.
3. Thiberge, N. F., Is oral pollen therapy dependable? South. Med. J., 1945, 38, 523.
4. Harsh, G. E., and Huber, H. L., Studies on tryptic and peptic digestion of extracts of giant ragweed pollen. J. Allergy, 1943, 14, 121.
5. Bozicevich, J., Studies on trichinosis, XII. The preparation and use of an improved trichina antigen. Pub. Health Rep., 1938, 53, 2130.
6. Rockwell, G. E., The effects of enzymes on ragweed pollen and studies of the iso-electric point of low ragweed antigen. J. Immunol., 1941, 41, 225.
7. Suessenguth, H., and Kline, B. S., A simple rapid flocculation slide test for trichinosis in man and in swine. Am. J. Clin. Path., 1944, 14, 471.
8. Jadassohn, W., Allergiestudien bei ascaredinidie synkarasie. Arch. f. Dermat. u. Syph., 1928, 156, 690.
9. Thiberge, N. F., The effect of gastric digestion on the allergic power of pollen. New Orleans M. and S. J., 1940, 92, 430.
10. Coca, A. F., and Grove, E. F., The nature of the pollen atopen. Proc. Soc. Exper. Biol. and Med., 1938, 21, 48.
11. Black, J. H., Studies in pollen sensitization. J. Lab. and Clin. Med., 1924, 10, 378.
12. Black, J. H., The oral administration of pollen. J. Lab. and Clin. Med., 1927, 12, 1156.
13. Melcher, L. R., An antigen analysis of *Trichinella spiralis*. J. Infect. Dis., 1943, 73, 31.
14. Bachman, G. W., A precipitin test in experimental trichiniasis. J. Prev. Med., 1928, 2, 36.
15. Kaljus, W. A., On the practical value of the intradermal reaction with the trichinelliasis antigen for the diagnosis of trichinelliasis in man. Puerto Rico J. Pub. Health and Trop. Med., 1936, 11, 768.
16. Roth, H., Serodiagnosis in trichinosis by microscopical testing with living trichina larvae. Nature, 1945, 155, 758.

PAMAQUINE NAPHTHOATE AS A PROPHYLACTIC FOR MALARIAL INFECTIONS [1]

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Plasmoquine, 6-methoxy-8-(1-methyl-4-diethylamino)butyl-aminoquinoline, has been assigned the name "Pamaquine" by the United States Pharmacopea XII, and will be so referred to in this presentation. This anti-malarial has been so frequently discussed in the literature that a detailed review of its history will not be included here. Reference will be made only to those papers which bear directly upon the subject of this report.

In 1931, James *et al* (1, 2) conducted a series of experiments which showed that pamaquine in doses of 80 mgm. per day on a 1-1-6 regime (that is, administered on the day before, the day of, and for 6 days after inoculation with sporozoites) acted as a true causal prophylactic for both *vivax* and *falciparum* inoculations (Roumanian strain). This work is summarized in Table I. It will be noted that the number of subjects tested was small. In view of this fact, and because of the importance of the results obtained, it is surprising that despite the reputation for toxicity which the drug had acquired, it was not explored further until a decision was made by the Panel on Clinical Testing of Anti-malarials of the Committee on Medical Research to reevaluate this drug. This project was assigned the task of repeating and expanding the study of James during the summer of 1944. The information obtained to date forms the basis of this report.

MATERIALS AND METHODS

(*a*) *Drug.* Pamaquine naphthoate was the compound employed throughout this study. Approximately 45 per cent of this salt is considered to be active base. Consequently, dividing our doses by 2 yields a figure for comparison with doses generally referred to in the literature. The original lot of drug employed was supplied in the form of 20-mgm. tablets. A second lot came as powder, and was capsulated locally. Both drug lots had such similar assays that they may be considered identical.

(*b*) *Plasma concentrations.* The plasma concentration of pamaquine was usually determined daily by means of a macrocolorimetric method (3) devised by Brodie *et al.*[6]

(*c*) *Subjects.* Thirty-six white and colored males and females, who were relatively normal except for central nervous system syphilis, were employed in these experiments. Their ages ranged from 14 to 60 years. Each received complete physical, hematological, and blood chemistry examinations, urinalysis, x-rays of the chest, and electrocardiographic studies prior to the institution of treatment. Subsequently, these tests (except for the roentgenograms) were performed daily until experience indicated that with the exceptions of determinations of methemoglobin, plasma bilirubin, hematocrit indices, and leukocyte counts, such extensive tests were unnecessary, and they were discontinued as routine procedures.

(*d*) *Methemoglobin.* Methemoglobin formation is known to occur regularly during pamaquine administration. Since this may be a factor in the serious toxic reactions ascribed to the drug, it was considered necessary to determine its concentration daily. These determinations were performed by means of a simple procedure devised by Wendel (4). The test utilizes a photoelectric colorimeter, and requires only small amounts of blood.

(*e*) *Parasitological methods.* Inoculations were generally performed on the second day of treatment, although exceptions to this rule will be noted in the tabulated data. Laboratory-bred *Anopheles quadrimaculatus,* infected by feeding on patients with either *P. vivax* (McCoy) or *P. falciparum* (Costa) parasites, were allowed to inoculate by feeding. The mosquitoes were then killed, dissected, and the sporozoite content of their salivary glands estimated by a scale of pluses ranging from 1 (minimal) to 4 (maximal). Although in obvious excess, a goal of 16 pluses was sought for each infection. When necessary (and available) additional mosquitoes were fed on the same subject if the initial inoculum was less than 12 pluses. Ordinarily, *P. vivax* was reserved for whites and *P. falciparum* for negroes. However, it was sometimes necessary to use *P. falciparum* in whites, but no mishaps occurred.

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[1] Work done under contract with the Office of Scientific Research and Development.

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TABLE I*

Pamaquine as a true causal prophylactic (Summary of the experiments of Col. James)

Group	Number of cases	Doses**	Duration of administration		Protection afforded (*i.e.*, no evidence malaria)			
			Total	Days after day of biting	Immediate (up to 30 days)		Late (followed up to 3 years)	
		grams	*days*		*no.*	*per cent*	*no.*	*per cent*
				P. vivax				
I	Not given	0.08 (0.08 first 3 days, 0.06 last 5 days)	8	6	All	100	All	100
II	10	0.06	7	5	10	100	5	50
III	5	0.04	10	8	5	100	Not tested	
IV	2	0.04	7	5	1	50	Not reported	
V	4	0.04	5	3	2	50	Not reported	
VI	5	0.04	4	2	1	20	Not reported	
VII (Roumanian strain)	3	0.08 evening before infection; 0.08 at time of infection (no other doses)			2	67	0	0
				P. falciparum				
I	Not given	0.08 (0.08 first 3 days, 0.06 last 5 days)	8	6	All	100	All	100
VIII (Roumanian strain)	5	0.08	6	4	5	100	5	100

* From a summary prepared by Dr. Thomas C. Butler for the Board for the Coordination of Malarial Studies.
** Presumed to be base, and thus equivalent to about 45 per cent of the naphthoate.

Each patient had a pretreatment negative thick film examination for malaria parasites. Similar smears were examined daily from the seventh postinoculation day until discharge from the hospital. All negatives were followed for at least 30 days in the hospital, and at intervals thereafter, depending upon the location of the individual's home or place of custodial hospitalization. Prior to the employment of penicillin for the treatment of the patients' neuro-syphilis, 3 subjects received trophozoite inoculations after 31 to 34 days of negative observation. The malarial course of each of these was perfectly normal.

Although it had been shown that penicillin had no effect upon malaria trophozoites (5, 6), no information was available concerning its action on sporozoite or "exoerythrocytic" forms. Consequently, 2 patients were given, in divided doses, 240,000 units of penicillin on each of 10 days. On the second day, one patient was inoculated by mosquitoes with sporozoites of *P. vivax* (McCoy), and the other with *P. falciparum* (Costa). Both developed malarial infections within normal prepatent periods, and so it was felt that the subsequent expression of infections in our experimental subjects was not influenced by intensive penicillin therapy received during the early prepatent phase.

RESULTS

The results of the experiments will be presented in relation to each of the parasite species employed, and in order of increasing drug dosage. This is not the chronological order of experimentation, but probably represents a more rational approach for the reader.

(*a*) *Experiments with* P. vivax (*McCoy*). The trials with *P. vivax* (McCoy) are summarized in Table II. Two patients who received 160 mgm. of pamaquine naphthoate a day for the second to sixth postinoculation days had prepatent periods of 15 and 19 days. Although slightly longer than usual, these periods are not significantly different from those normally encountered. This experiment was designed to test the thesis that pamaquine acted against other than sporozoite and trophozoite forms. The results in these 2 patients do not indicate that such, at least, is the sole case.

In order to determine whether the activity of pamaquine depended upon a slowly excreted degradation product, 180 mgm. were administered daily for 8 days to 1 patient. Inoculation was accomplished on the ninth day, and 12 days later he developed a demonstrable parasitemia. In this instance, at least, the hypothesis appears to have been unfounded.

Another patient, receiving 180 mgm. of pamaquine naphthoate a day, was inoculated on the second of the 2 days in which he received this dose. The appearance of an acute hemolytic anemia then necessitated drug withdrawal; parasitemia was evident on the eleventh postinoculation day.

One patient was given 160 mgm. of drug on the day before, the day of, and for 2 days following inoculation. The development of severe abdominal pain necessitated stoppage of the medication. He developed malaria on the eighteenth postinoculation day.

One patient was inoculated on the fourth of an 8-day course of 180 mgm. of pamaquine naphthoate daily. This was considered to represent a plasma mixture of "fresh" and "degraded" drug. Malaria was apparent on the twenty-third day, somewhat later than the normally expected date.

One subject received 80, and another 120 mgm. of pamaquine naphthoate daily on the day before, the day of, and for 5 days following inoculation. The former developed malaria on the fourteenth, and the latter on the sixteenth day. In view of other similar experiments (*vide infra*) these dosages appear to have been indaequate.

Another patient was given 160 mgm. of pamaquine naphthoate daily on the same schedule as the 2 patients just described. After 34 negative days he received an intravenous inoculation of *P. vivax* (McCoy) trophozoites, and a normal malarial course ensued. Two hundred and thirty days later he was admitted for a malarial attack. Since blood-induced infections do not ordinarily relapse, this probably represents a break-through of the original sporozoite inoculation, but the slight factor of uncertainty eliminates this patient from further consideration.

Five patients were administered 160 mgm. of pamaquine naphthoate for each of 3 days, and then 120 mgm. daily for 5 days. Inoculation was per-

TABLE II

Summary of prophylaxis trials with pamaquine naphthoate vs. P. vivax *(McCoy)*

Drug schedule	Patient	Race	Age	Wgt.	Dosage	Number days	Number doses	Mg. per kg.*	Total dose	Sporozoite doses**	Prepatent days	Days neg. obs.	Remarks
			yrs.	kgm.					grams				
0-0-0-5***	SHE	W	32	77	40 mgm. q. 6 h.	5	20	2.1	0.8	14	15		{Drug during 2nd–6th
	JOH	W	40	66		5	20	2.4	0.8	15	19		post-inoc. days
8-0-0	BYR	W	50	65	22.5 mgm. q. 3 h.	8	64	2.8	1.44	12	12		
1-1-0	JOR	C	38	55	22.5 mgm. q. 3 h.	2	16	3.3	0.36	19	11		Hemolytic anemia
1-1-2	STA	W	26	58	40 mgm. q. 6 h.	4	16	2.8	0.64	20	18		Severe abd. pain
3-1-4	FER	W	24	74	22.5 mgm. q. 3 h.	8	64	2.4	1.44	16	23		
1-1-5	STE	W	40	83	20 mgm. q. 6 h.	7	28	1.0	0.56	20	14		
	BAK	W	25	64	20 mgm. q. 4 h.	7	42	1.9	0.84	20	16		
	PHI	W	39	65	40 mgm. q. 6 h.	7	28	2.5	1.12	16		34	Troph. inoc. 35th day
1-1-6	MOO	W	24	80	{40 mgm. q. 6 h.,	8	40	2.0	1.04	17	248		
	WIL	W	49	71	× 12, 20 mgm.,	8	40	2.3	1.04	16	194		
					q. 4 h. × 28								
	POR	W	35	60	{40 mgm. q. 6 h.	8	42	2.7	1.08	18		445	
	LEW	W	50	74	× 12, 20 mgm.	8	42	2.2	1.08	16		443	
	HAL	W	34	69	q. 4 h. × 30	8	42	2.3	1.08	20		413	
	BAS	W	27	69		8	64	2.6	1.44	17	22		
	JER	W	44	50		8	64	3.6	1.44	18	262		
	McC	W	14	60	22.5 mgm. q. 3 h.	8	64	3.0	1.44	17	209		
	SIM	W	41	74		8	64	2.4	1.44	19		121	
	BEA	W	29	65		8	64	2.8	1.44	18		187	

* Where combinations of doses were employed, this figure was based upon the amount of drug administered during the first day.

** Sporozoite content of salivary glands of infecting mosquitoes estimated by scale of pluses ranging from one (minimal) to four (maximal). This column represents total for the individual patient.

*** The first number refers to the preinoculation period, the second, to the day of inoculation, and those that follow to the postinoculation period.

formed on the second day. None of this group suffered an immediate primary attack. One had a delayed primary episode at 194 days, another at 248 days, while the remaining 3 have now been followed for 413, 443, and 445 days respectively, without demonstrable evidence of malaria. Since no untreated patient on this service has ever failed to develop malaria within less than 20 days after mosquito inoculation, these results are of considerable significance.

Five patients received 180 mgm. of pamaquine naphthoate daily for 8 days with inoculation on the second day. One of these developed clinical malaria on the twenty-second day, another at 209 days, and the third at 262 days. The other 2 have not developed demonstrable infections during 121 and 187 observation days, respectively.

In view of the small numbers of subjects dealt with, no attempt will be made to explain the differences in the results obtained in the last 2 groups.

Four random-selected, untreated patients who received similar *P. vivax* (McCoy) inoculations at various times during this study had prepatent periods of 11 to 16 days.

(*b*) *Experiments with* P. falciparum (*Costa*). The experiments with this species are summarized in Table III.

One patient received 160 mgm. of pamaquine naphthoate on the day of inoculation only, and developed malaria 11 days later. The mosquitoes were fed midway during the drug course. In another instance, inoculation occurred after the administration of 157.5 mgm. of pamaquine naphthoate, when the drug had to be stopped because of what was interpreted as a severe lumbar puncture reaction. Malaria was evident 9 days later.

Of 2 patients who received 160 mgm. of drug a day for 3 days (inoculation on the second day), one had a prepatent period of 14 days, whereas the other has been negative for 489 days. The latter received the smallest sporozoite dose (8+) of any patient in our series, but such inocula ordinarily are sufficient to produce infection.

One subject, whose drug had to be withdrawn after 4 days on the 160 mgm. schedule because of an acute hemolytic anemia, developed malaria on the thirteenth day. Inoculation, as usual, was done on the second day.

TABLE III*

Summary of prophylaxis trials with pamaquine naphthoate vs. P. falciparum (*Costa*)

Drug schedule	Patient	Race	Age	Wgt.	Dosage	Number days	Number doses	Mgm. per kgm.	Total dose	Sporozoite dose	Prepatent days	Days neg. obs.	Remarks
			yrs.	*kgm.*					*grams*				
0-1-0	BAK	W	25	68	40 mgm. q. 6 h.	1	4	2.4	0.16	26	11		
1-0-0	CIT	W	33	59	22.5 mgm. q. 3 h.	1	7	3.1	0.157	15	9		Drug stopped. LP Reaction
1-1-1	COO	C	19	57	40 mgm. q. 6 h.	3	12	2.8	0.48	8		489	
	SLA	W	52	60		3	12	2.7	0.48	27	14		
1-1-2	BRO	C	41	51	40 mgm. q. 6 h.	4	15	3.0	0.6	21	13		Hemolytic anemia
1-1-3	SMI	W	42	60	22.5 mgm. q. 3 h.	5	40	3.0	0.9	20		226	
4-1-3	MOR	C	48	60	22.5 mgm. q. 3 h.	8	64	3.0	1.44	11		254	
1-1-4	JAC	C	49	63	22.5 mgm. q. 3 h.	6	48	2.9	1.08	24		232	
	HOL	C	44	53		6	48	3.4	1.08	17		158**	
1-1-5	LEF	C	33	48	40 mgm. q. 6 h.	7	28	3.3	1.12	14		31	Troph. inoc. 32nd day
	JON	C	36	42		7	28	3.8	1.12	14		31	Troph. inoc. 32nd day
	BUT	C	14	53		7	28	3.0	1.12	16		483	
-1-6	WEL	C	31	60	40 mgm. q. 6 h. × 12, 20 mgm. q. 4 h. × 30	8	40	2.7	1.04	13		455	
	RIN	W	40	72		8	42	2.2	1.08	21		349	
	DAV	C	30	61	22.5 mgm. q. 3 h.	8	64	3.0	1.44	20		245	
	PRO	W	34	69		8	64	2.6	1.44	20		181	
	ScH	W	45	54		8	64	3.3	1.44	20		221	

* Table II footnotes apply to Table III.

** Died subsequently of non-malarial condition.

TABLE IV

Plasma concentrations of pamaquine expressed as gammas per liter

Mgm. per day	Patient*	Day 1**	2	3	4	5	6	7	8	9	10	11	Ave.
160	BRO	—	141	180	180	270***	33	—	24	0			138
	SLA	—	75	52	52	270	0						112
	JOH	0	52	37	—	88	81	—					65
	SHE	—	52	37	—	78	75	—					61
	STA	—	60	90	90	150***	0						98
160 + 120	HAL	—	36	52	70	40	33	33	30	33	0		41
	LEW	0	82	120	165	75	82	381	—	60	30	0	124
	MOO	0	30	52	30	—	75	36	150	30	0		58
	POR	0	33	24	52	30	37	36	60	30	0		38
	RIN	0	33	—	36	33	10	24	30	30	0		25
	WEL	0	82	82	105	—	120	33	36	30	0		70
	WIL	0	—	—	180	180	165	138	150	24	0		140
180	BAS	0	81	75	—	45	157	81	162	75	0		97
	BEA	0	81	135	141	135	—	141	150	165	0		135
	BYR	—	78	—	138	165	165	165	270	210	—	0	170
	CIT	—	45	0									45
	DAV	0	790	1,000	1,320	—	1,440	1,440	1,200	1,190	330	180	1,197****
	FER	0	75	81	81	78	—	114	150	135	0		102
	HOL	—	150	90	81	150	135	135	—	0			124
	JAC	0	180	—	135	286	243	286	—	0			226
	JER	0	78	—	150	135	135	141	165	81	0		126
	JOR	0	81	135***	—	0							108
	MOR	0	231	237	189	165	—	286	243	210	45	0	201
	PRO	0	60	45	60	—	105	120	160	82	0		90
	ScH	0	82	82	120	—	160	135	125	135	0		120
	SMI	0	150	264	150	135	135	0					167
	SIM	0	60	60	60	95	—	105	50	115	0		78
	McC	0	82	105	60	60	—	82	40	135	0		81

* Identification corresponds to Tables II and III.
** The day drug administration was begun but prior to the first dose. — not done, 0 no drug detectable in plasma.
*** Drug stopped.
**** Determination of plasma concentration not done on 12th day and not detectable on 13th day.

In the next group, both patients received drug for 3 days following the day of inoculation, but whereas one had been treated for 3 days prior to that date, the other received the drug for only 1 day before inoculation. The dose was 180 mgm. a day. Neither has developed malaria during 254 and 184 days of observation.

Two others who received 180 mgm. of pamaquine naphthoate on the day before, the day of, and for 4 days following inoculation, have been free of disease for 158[7] and 189 days, respectively.

Of 3 patients who received 160 mgm. of drug for 7 days (inoculated on the second day), 2 were given trophozoites intravenously after 31 days of negative observation, and so have been dropped from the study, while the third has been free of malaria for 483 days.

Two subjects have had no demonstrable malaria for 349 and 455 days following a drug schedule of 160 mgm. a day for 3 days, followed by 120 mgm. for each of 5 days. Inoculation was accomplished on the second day.

Three patients who were on the 8-day, 180 mgm. daily schedule have not developed malaria during 245, 181 and 221 days, respectively.

A representative group of 6 untreated patients who received *P. falciparum* (Costa) sporozoites by the same inoculation method while these studies were in progress had prepatent periods of 8 to 12 days.

(*c*) *Plasma pamaquine levels.* The plasma concentrations of pamaquine determined in 28 patients are summarized in Table IV. These demonstrate quite clearly that measurable pamaquine is both rapidly absorbed into, and excreted from, the plasma. However, it is just as apparent that the plasma level attained (as measured by

[7] Died subsequently of non-malarial condition.

TABLE V

Methemoglobinemia as per cent of total hemoglobin in patients receiving pamaquine naphthoate

Mgm. per day	Patient*	Day 1**	2	3	4	5	6	7	8	9	10	11	12	13	14	15
160	BRO	—***	2	4	13	17	3	—	2	1	—					
	SLA	—	2	2	5	5	3	—								
	HAL	—	2	2	3	5	3	—	6	6	7	—				
	JOH	4	4	6	—	9	11	10	—							
	LEW	3	3	5	5	8	9	11	—	12	11	—				
	MOD	1	2	3	2	—	4	4	4	2	8	3	—			
	POR	2	2	—	2	5	6	7	7	6	—	6	—			
	RIN	1	1	2	2	2	—	3	3	3	2					
	SHE	3	3	6	—	5	8	7	—							
	STA	—	1	—	8	14	19	16	—	13	8	10	—	—	6	—
	WEL	1	2	2	2	—	3	3	3	3	9	3	—			
	WIL	1	2	—	4	5	5	7	7	7	—					
180	BAS	3	5	6	—	10	10	10	—	10	9	—				
	BEA	—	3	5	7	9	—	15	16	19	14	—				
	BYR	—	3	5	7	10	13	15	17	13	—					
	CIT	—	1	2	—											
	DAV	3	3	5	10	—	18	20	24	22	19	20	—	—	16	—
	FER	2	3	3	4	5	—	7	8	10	10	—				
	HOL	—	2	2	6	4	8	14	—	6	—					
	JAC	—	—	4	6	8	13	12	—	12	—					
	JER	3	5	—	5	10	12	14	16	16	—	14	—			
	JOR	4	5	8	—	10	—									
	MOR	2	3	6	9	—	8	9	15	—	7	—				
	PRO	3	3	5	9	—	14	16	20	18	19	—				
	ScH	3	2	3	7	—	12	12	13	14	14	—				
	SMI	—	3	5	7	12	7	—	12	—						
	SIM	—	2	2	3	4	—	8	7	8	8	—				
	McC	—	2	2	3	5	—	9	8	11	8	—				

* Identification corresponds to Tables II and III.
** The day drug administration was begun, but prior to the first dose.
*** Not done.

the method employed) bears little relationship to the mgm. per kgm. dose, the various toxic reactions produced, or to the degree of protection attained. In general, plasma concentrations were greater in those whose total drug intake was greater. This is illustrated by the averages for the dosage groups.

(*d*) *Toxic reactions.* Although methemoglobinemia was regularly encountered at all dosage levels (Table V), more tended to be formed as the pamaquine intake was increased. Such formation occurred quite soon after the initiation of therapy, and persisted for some days after the drug was withdrawn. No methylene blue could be employed for the treatment of this state because of its effect upon malarial parasites. Of itself, the methemoglobinemia did not appear to cause any difficulty, although high levels were attained in individuals who suffered other severe toxic reactions.

Two instances of acute hemolytic anemia were encountered. Both occurred in negro patients, and both had their onset soon after therapy was begun. In neither instance was there any history of preceding malaria or exposure to pamaquine. Prompt withdrawal of the drug and intensive erythrocyte transfusions produced complete recoveries. The data concerned with these 2 patients are summarized in Tables VI and VII. In addition, Table VII includes a summary of attempts to reproduce this hemolysis *in vitro*. All of the latter were failures.

An opportunity to restudy patient BRO presented itself some 3 months after the initial hemolytic episode. At this date he was fully recovered and normal in all respects. He was placed on a drug schedule which called for 160 mgm. of pamaquine naphthoate a day for 3 days, followed by 120 mgm. for each of 5 following days, but the drug had to be withdrawn on the fifth day because of another hemolytic reaction (Table VIII). This indicates that the susceptibility to acute he-

TABLE VI

Hemolytic reaction to pamaquine naphthoate

Patient: JOR. Negro, male. *P. vivax* (McCoy)

Date	Hour	Drug	Plasma level	Hemato-crit	Plasma bilirubin	Total hb.	Per cent met-hb.	Toxic manifestations	Remarks
			γ per l.		mgm. per cent	grams per cent			
7-19	8:30 a.m.	**	0	—	—*	14	4		
	12:00 p.m.	Started							
7-20	8:30 a.m.		81	38	1.21	12.8	5		19 + Sporozoites
7-21	8:30 a.m.	Stopped	135	37.5	3.65	11.1	8	Weakness	
7-22	8:30 a.m.		—	28.5	3.71	—	—	Headache, dizziness	
7-23	8:30 a.m.		0	21	2.61	9.6	7	Dizziness, nausea, and vomiting	
	2:30 p.m.		—	28	2.2	—	—		1 hour after 400 ml. RBC IV
	7:30 p.m.								600 ml. RBC IV
7-24	8:30 a.m.		—	32.5	3.6	—	—	None	
7-25	8:30 a.m.		—	34	1.21	—	—		
7-27	8:30 a.m.			32.5	0.23	—	—		

* Not done.

** Mgm. 22.5 q. 3 h. × 16.

molytic reactions because of pamaquine intake persists for 3 months at least.

Since the question of the role of various blood grouping factors in the production of this type of hemolytic reaction might be raised, serum samples obtained from patients BRO and JOR (as well as from several other individuals who had received similar amounts of the same drug with-

TABLE VII

Hemolytic reaction to pamaquine naphthoate (first course)

Patient: BRO. Negro, male. *P. falciparum* (Costa)

Date	Hour	Drug	Plasma level	Hemato-crit	Plasma bilirubin	Total hb.	Per cent met-hb.	Toxic manifestations	Remarks
			γ per l.		mgm. per cent	grams per cent			
2-12	8:30 a.m.	**	0	—*	—	—	—		
	12:00 p.m.	Started							
2-13	8:30 a.m.		141	45	—	14.4	2		21 + sporozoites
2-14	8:30 a.m.		180	43	—	14.2	4		Plasma slightly discolored
2-15	8:30 a.m.		180	38	2.4	13.1	13	Slight abdominal pain	
	***								
2-16	8:30 a.m.		270	31	3.4	10.1	17	Slight abdominal pain	
	2:00 p.m.	Stopped							Total, 0.58 gm.
2-17	8:30 a.m.		33	25	3.0	7.7	3	Abd. pain, weakness, pallor	
	2:30 p.m.		—	33	4.9	9.6	—	Abd. pain, weakness, pallor	1 hour after 400 ml. RBC IV
2-18	10:30 a.m.		—	30	3.9	7.8	—	Abd. pain, weakness, pallor	Followed by 600 ml. RBC IV
2-19	8:30 a.m.		24	37	2.2	10.8	2	Slight abd. pain	Clinical recovery
2-20	8:30 a.m.		0	34	0.6	10.7	1	None	
2-23	8:30 a.m.			38	—	—	—		

* Not done.

** 40 Mgm. q. 6 hrs. × 12, then 20 mgm. q. 4 hrs. × 5.

*** (a) Erythrocyte fragility—normal limits. (b) BRO's washed (3× in PSS) RBC suspended in homologous plasma and in plasma of control of same blood group (who had had similar pamaquine course 2 weeks previously without mishap) in concentration of hematocrit for this day, incubated in 37° C. H_2O bath: No hemolysis in 16 hours. (c) As in (b) but with pamaquine HCl added to control plasma in concentrations of 100, 200, 400, 1,600, 3,200 gammas per liter: No hemolysis in 16 hours. (d) RBC of control (0) receiving pamaquine plus BRO's plasma (0): No hemolysis after 16 hours at 37° C. Thus, *in vivo* hemolysis of BRO's RBC not reproducible *in vitro* by these studies.

TABLE VIII

Hemolytic reaction to pamaquine naphthoate (second course)

Patient: BRO. Negro, male

Date	Hour	Drug	Plasma level	Hematocrit	Plasma bilirubin	Per cent met-hb.	Toxic manifestations	Remarks
			γ per l.		mgm. per cent			
5–14	8:30 a.m.	**	0	38.5	0.35	—*	None	
	12:00 p.m.	Started						
5–15	8:30 a.m.		360	35.5	0.35	4		
5–16	8:30 a.m.		360	—	0.75	3		
5–17	8:30 a.m.		420	33	1.04	4	Diarrhea mild. Moderate weakness	No transfusion administered
5–18	8:30 a.m.		300	33.5	1.21	4		
5–19	8:30 a.m.		290	29.5	1.10	4		
	10:00 a.m.	Stopped						
5–21	8:30 a.m.		0	36	0.46	5		
5–22	8:30 a.m.		—	32.5	0.29	2		

* Not done.

** Mgm. 40 q. 6 hrs. × 12, then mgm. 20 q. 4 hrs. × 13.

out any untoward reaction) were coded and forwarded to Dr. Philip Levine for analysis. The results of his studies appear in Table IX. It will be noted that none of these grouping factors, single or in combination, appear to be related to hemolytic susceptibility to pamaquine.

TABLE IX

Analysis of individual blood differences of six patients who received pamaquine naphthoate with and without hemolytic reactions*

Patient	Toxic reaction	Group	MN	Anti-Rh_0	Anti-Rh'	Anti-Hr	Rh type
BRO	Hemolytic	A (A_1)	M	+	o	+	Rh_2
JOR	Hemolytic	B	MN	+	+	+	Rh_1
STA	Abdominal pain	O	MN	o	o	+	Rh negative
BUT	None	O	M	+	o	+	Rh_2
PRO	None	O	MN	o	o	+	Rh negative
ScH	None	A (A_2)	MN	+	+	+	Rh_1

* Performed by Dr. Philip Levine, Ortho Research Foundation, Linden, New Jersey.

Abdominal pain occurred in 4 patients. One of these (STA) had such a severe episode that the drug had to be withdrawn (Table X). The pain was of a griping character, and was concentrated in the umbilical area. He also complained of moderate headache and nausea. The other 3 had short bouts of mild pain following some of the drug doses, but all were able to complete their schedules without mishap.

Of 26 patients (omitting the 2 hemolytic reactors), 11 had increases in serum bilirubin (7) of more than 0.5 mgm. per cent. These changes represent the differences between samples examined prior to, and at, the completion of treatment. Seven of these individuals were concentrated in the 11 patients who received 180 mgm. of pamaquine daily for 8 days.

Fourteen of 27 patients (omitting the 2 hemolytic reactors) had decreases in their hematocrit indices of the order of 3 or more. All 14 occurred among the 18 patients who received drug for 8 days.

The total leukocyte counts diminished by 1 thousand or more in 11 of 27 patients, remained unchanged in 9, and increased by 1 thousand or more in 7 others. Two of the 11 decreases finished with counts of 3 to 4 thousand per cubic millimeter.

Neither drug fever nor urinary abnormalities were encountered in any of the 36 patients.

COMMENTS

The data demonstrate quite clearly that when either 160 or 180 mgm. of pamaquine naphthoate are administered on the day before, the day of, and for 5 or 6 days (1-1-5 or 1-1-6) following sporozoite inoculation with *P. vivax* (McCoy), clinical expression of the disease may be delayed, or prevented, for months. Similar results may be anticipated with as few as 3 postinoculation days (1-1-3) treatment when *P. falciparum* (Costa) is the test parasite employed.

TABLE X

Severe abdominal pain necessitating withdrawal of pamaquine naphthoate
Patient: STA. White, male. *P. vivax* (McCoy)

Date	Hour	Drug	Plasma level	Hemato-crit	Plasma bilirubin	Per cent met-hb.	Toxic manifestations	Remarks
			γ *per l.*		*mgm. per cent*			
11–28	8:30 a.m.	**	0	44	0.7	—*		
	12:00 p.m.	Started						
11–29	8:30 a.m.		60	45	0.9	1		20 + sporozoites
11–30	8:30 a.m.		90	—	—	—	Slight abdominal pain, tinnitus	
12–1	8:30 a.m.		90	44	0.2	8	Moderate abdominal cramps	
12–2	8:30 a.m.		150	46	—	14	Severe cramps, nausea, headache, cyanosis	
	2:00 p.m.	Stopped						Total, 0.68 gram
12–3	8:30 a.m.		0	44	0.2	18	Slight cyanosis	All symptoms relieved 12 hrs. after drug withdrawn
12–4	8:30 a.m.		0	43	0.4	16		

* Not done. ** 40 mgm. q. 6 hrs. × 12, then 20 mgm. q. 4 hrs. × 7.

This is quite different from experiences with quinacrine (atabrine), for one achieves suppression (*P. vivax*) or suppression-until-cure (*P. falciparum*) with this compound only if its administration is continued through the period of the expected primary attack. A further indication of the differences between the mode of action of pamaquine and atabrine or quinine is demonstrated by the effect of combining pamaquine with 1 of the other 2 for the treatment of vivax relapses. Such combined therapy will usually prevent further relapses. Dixon's study (8), while not the first of its kind, illustrates this point quite clearly.

Our study also highlights again the fact that falciparum and vivax parasites differ considerably in their susceptibility to antimalarial drugs. Whether this means that the 2 have such different life cycles that a drug must have a particular form to affect adversely one species and not the other, is pure conjecture.

Although our experiments failed to demonstrate as complete protection as was achieved by James in his trials with the Roumanian strains, nor, on the other hand, were the attained results as poor as those reported by Alving *et al* (9) with the markedly relapsing Chesson strain of South Pacific vivax, they did accomplish their basic purpose, which was to determine whether James's results could be supported sufficiently to warrant an extensive chemical synthesis program to find other, less toxic, more active pamaquine analogs.

Alving *et al* employed doses of 90 mgm. of active pamaquine base per day on a 1-1-6 regime against the Chesson vivax strain in 5 subjects. Three of these patients developed malaria after 18, 19, and 106 days, whereas the remaining 2 have been observed for 402 negative days. These results, coupled with those of James and ourselves, illustrate again the danger of generalizing upon the subject of antimalarial therapy from experiences with a single strain of parasites.

SUMMARY AND CONCLUSIONS

1. Pamaquine naphthoate was administered to 36 paretics as a prophylactic against mosquito-induced infections with *P. vivax* (McCoy) and *P. falciparum* (Costa).

2. Daily doses of either 160 or 180 mgm. administered the day before, the day of, and for 5 or 6 days following the day of inoculation with *P. vivax* (McCoy) have prevented or postponed the primary attack for months.[8] In the case of *P. falciparum* (Costa) postinoculation treatment could be shortened to 3 days with similar results,

[8] *Addendum:* 15 January, 1947. The number of days of negative observation, through this date, for those patients still available for follow-up, were as follows:
P. vivax. POR, 690; LEW, 728; HAL, 648; BEA, 410.
P. falciparum. COO, 774; SMI, 340; BUT, 670; WEL, 465; RIN, 543; DAV, 345; PRO, 348; ScH, 474.

except that delayed primary attacks were not observed.

3. Two instances of acute hemolytic anemia were encountered among 11 negro patients. One of these was re-exposed to the drug, and promptly had another hemolytic episode. This type of reaction was not related to the recipient's blood group.

4. Methemoglobinemia was regularly encountered in all subjects.

5. Elevations of the serum bilirubin and moderate hematocrit decreases were commonly noted among those receiving the higher, more prolonged courses, regardless of race.

6. Pamaquine naphthoate is rapidly absorbed and excreted.

7. The mode of action of pamaquine as a malaria prophylactic or suppressive is not explained, although such activity appears to be a function of the parasite species (and strain) employed, the total amount of drug ingested, and the number of days the drug is administered in relation to the inoculation day.

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BIBLIOGRAPHY

1. James, S. P., Nicol, W. D., and Shute, P. G., On the prevention of malaria with plasmaquine. Lancet, 1931, 2, 341.
2. Quarterly Bulletin, Health Organization, League of Nations, 1933, 2, No. 2, June.
3. Brodie, B. B., Udenfriend, S., and Taggart, J. V., Analysis of basic organic compounds in biological tissues: 4. Coupling with diazonium salts. Fed. Proc., Part II, 1946, 5, 125.
4. Wendel, William B. To be reported.
5. Lyons, C., Penicillin therapy of surgical infections in the U. S. Army. J. A. M. A., 1943, 123, 16, 1007.
6. Hindle, J. A., Rose, A. S., Trevett, L. D., and Prout, C., The effect of penicillin on inoculation malaria. N. Eng. J. Med., 1945, 232, 5, 133.
7. Malloy, H. T., and Evelyn, K. A., The determination of bilirubin with the photoelectric colorimeter. J. Biol. Chem., 1937, 119, 481.
8. Dixon, H. B. F., A report on 600 cases of malaria treated with plasmoquine and quinine (Abst.). Trop. Dis. Bull., 1933, 30, 12, 846.
9. Alving, A. S., Eichelberger, L., Craige, B., Jr., Jones, R., Jr., Pullman, T. N., and Whorton, C. M., A study of the prophylactic effect of several 8-amino quinolines including plasmochin for South Pacific (Chesson) vivax malaria. Presented at the 38th Annual Meeting of the American Society for Clinical Investigation, Atlantic City, New Jersey, 27 May 1946. Abstracted in J. Clin. Invest., 1946, 25, 910.

ELECTROPHORETIC CHANGES IN THE SERUM OF A PATIENT WITH RHEUMATOID ARTHRITIS

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(Received for publication August 8, 1946)

Various reports in recent years (1 to 5) have indicated that changes occur in the serum electrophoretic patterns of patients with rheumatoid arthritis. The present study is designed to supplement these generally isolated measurements with serial observations of the changing serum pattern through a cycle of the disease, correlated with immunological and clinical data. Of interest also is the comparison of data from this clinically typical case of rheumatoid arthritis with the data, previously reported (6), from patients with rheumatic fever of comparable severity and duration.

METHODS

All measurements, common to those of the earlier study (6), were made in the same way.

The antifibrinolysin antibody (AFL) was determined by the method of Tillett, Edwards and Garner (7), as modified by Boisvert (8).

The C-reactive protein was measured by a modification of the method of Tillett and Francis (9): 0.1 ml. of undiluted test serum was mixed with 0.1 ml. solutions of the pneumococcus C polysaccharide,[1] varying in concentration downward from 10^{-4}. After incubation at 37° C. for 2 hours, the tubes were left overnight at 4° C., then read for precipitation. A known strongly positive serum from a patient with acute rheumatic fever was included as a control.

SUBJECT OF THE STUDY

C. B. (No. 11, 113), a 28-year old machinist, was admitted to another hospital on the 8th day following the onset of polyarthritis. Prior to this, he had always enjoyed good health, and in particular had had no previous joint symptoms. The present attack was not preceded by any apparent respiratory infection. During a 2-month stay at that hospital, he had persistent painful swelling of an ankle, a knee and the small joints of the hands, not appreciably relieved by salicylates. An elbow had become involved shortly before admission.

When admited to the Rockefeller Hospital on the 64th day of illness he appeared chronicaly ill; his weight had fallen to 55 kgm. from a normal of about 72 kgm. Examination of the heart revealed no abnormalities either on admission or subsequently. There was definite atrophy of the muscles of the hands, both legs and one forearm. X-ray examination showed decalcification of the bones and periarticular swelling without narrowing of the joint spaces of the hands and knees. Cultures of nose and throat revealed no hemolytic streptococci, and the gonococcus complement fixation and Wassermann tests were both negative.

During 8 months of hospitalization he gradually improved under treatment with initial bed rest followed by slowly increased activity; hot packs to the affected joints with short periods of active exercise and plaster support as required were also employed. Aspiration of the right knee joint on the 248th day yielded 30 ml. of yellow, turbid and viscid fluid which contained 5,300 leuckocytes per c.m.m. (49 per cent polynuclear, 45 per cent lymphocyte, 6 per cent monocyte). The culture was negative.

At the time of discharge on the 332nd day, he was capable of doing light work; the only residual joint symptoms were slight swelling with limited flexion of one knee and weakness of one finger.

OBSERVATIONS AND DISCUSSION

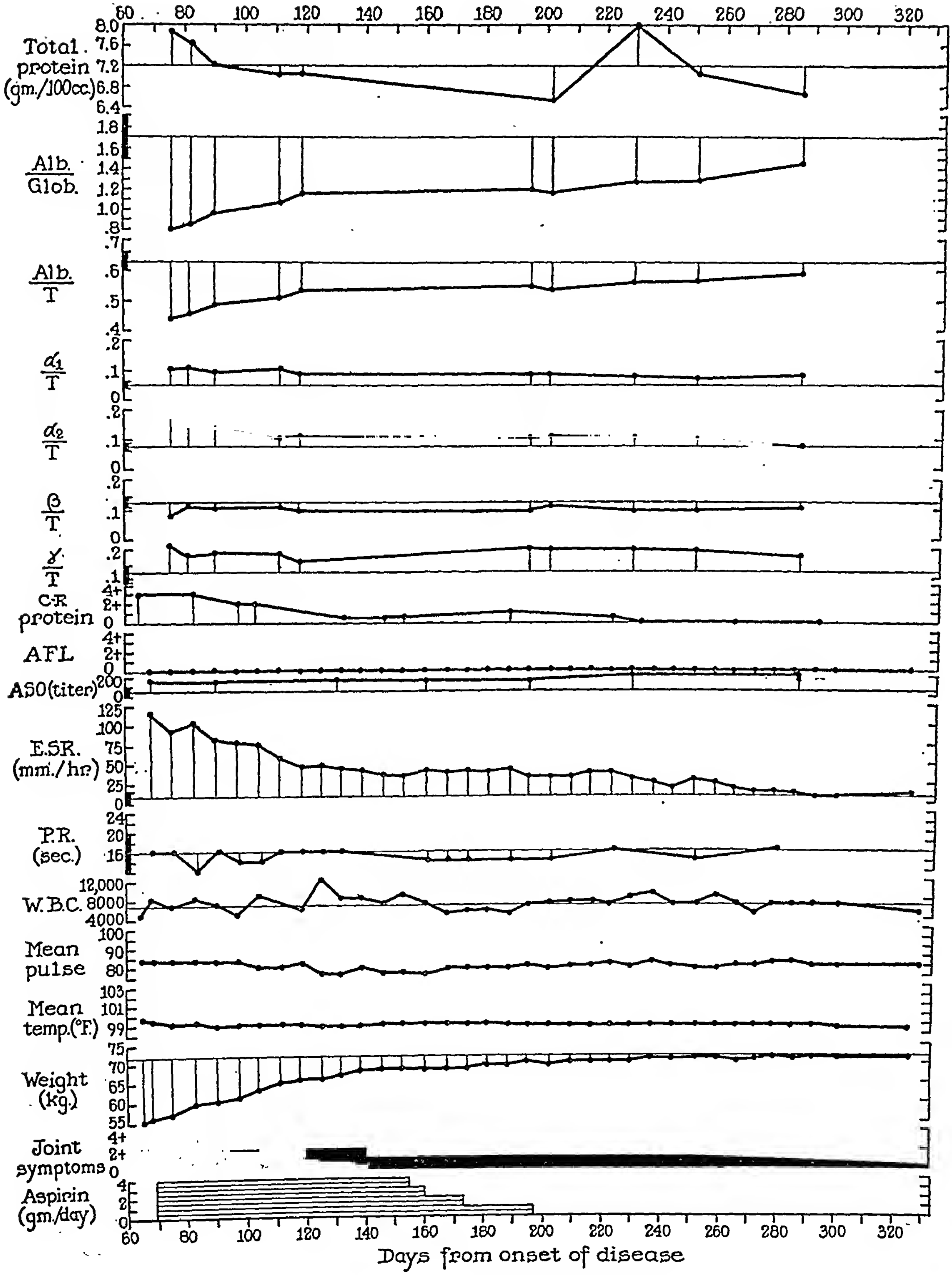
The total protein concentration was not systematically affected. The relative proportion of albumin, and with it the A/G ratio, was considerably depressed in the earlier observations, but later returned toward normal. Both α globulins were markedly elevated (over twice their normal values) during the early stages, then fell to essentially normal proportions. β globulin was on the low side of normal throughout the study. γ globulin maintained a fairly constant level about 35 per cent over the normal value.

These changes generally conform to those described by other authors (1 to 5), who have reported increases in the α and γ globulins, independently or in combination, and decreases in albumin.

A slight elevation above normal limits was observed in the titer of antistreptolysin O. According to other workers (10) such increases may occur in rheumatoid arthritis, but are exceptional. More typical of the disease is the absence of an antifibrinolysin response (11 to 13).

The C-reactive protein is an interesting protein of unknown function that has been detected in the

[1] Kindly supplied by Dr. Maclyn McCarty.


Total protein (gm./100cc.)
Alb. / Glob.
Alb. / T
α_1 / T
α_2 / T
β / T
γ / T
C-R protein
AFL
ASO (titer)
E.S.R. (mm./hr.)
P.R. (sec.)
W.B.C.
Mean pulse
Mean temp. (°F.)
Weight (kg.)
Joint symptoms
Aspirin (gm./day)
Days from onset of disease

serum during the early phases of diverse infectious diseases (14, 15, 9). It has not yet, however, been sufficiently studied to determine whether its appearance is confined to diseases of infectious etiology (16). The occurrence of this protein in association with increased α globulins accords with the findings of previous workers (17) that it is contained in the α_1 globulin fraction.

As might be expected, the serum protein pattern varied with change in the clinical status; it is possible that a sufficiently extensive study might reveal correlations of prognostic significance. It is clear, on the other hand, that electrophoretic measurement holds no diagnostic promise, since the present findings appear to be indistinguishable from those previously reported for rheumatic fever (6).

SUMMARY

1. Serial electrophoretic observations of the serum from a patient with rheumatoid arthritis showed a depressed proportion of albumin with increased relative amounts of α and γ globulins.

2. These abnormalities in serum protein pattern decreased with clinical improvement.

3. Antistreptolysin O antibody titer showed a low constant elevation. Antifibrinolysin activity was not detectable.

4. The C-reactive protein, which was present in high titer during the early acute phase of the disease, disappeared with clinical recovery.

BIBLIOGRAPHY

1. Svartz, N., Nord. Med., 1943, 18, 673. Nord. Med., 1944, 23, 1713. } in Malmros, H., and Blix, G. (4).
2. Olhagen, B., Om electroforese diagram pa plasma från homo vid patologiske tillstand. Nord. Med., 1944, 23, 1530.
3. Lövgren, O., Studien über den intermediären stoffwechsel bei chemischer polyarthritis. Acta Med. Scand., Suppl. 163, 1945
4. Malmros, H., and Blix, G., The plasma proteins in cases with high erythrocyte sedimentation rate. Acta Med. Scand., Suppl. 170, 1946.
5. Perlmann, Gertrude E., Kaufman, Dorothy, and Bauer, Walter., Electrophoretic distribution of proteins in serum, plasma and synovial fluid of patients with rheumatoid arthritis (to be published).
6. Dole, V. P., Watson, R. F., and Rothbard, S., Electrophoretic changes in the serum protein patterns of patients with scarlet fever and rheumatic fever. J. Clin. Invest., 1945, 24, 644.
7. Tillett, W. S., Edwards, L. B., and Garner, R. L., Fibrinolytic activity of hemolytic streptococci. The development of resistance to fibrinolysis following acute hemolytic streptococcus infections. J. Clin. Invest., 1934, 13, 47.
8. Boisvert, P. L., The streptococcal antifibrinolysin test in clinical use. J. Clin. Invest., 1940, 19, 65.
9. Tillett, W. S., and Francis, T., Jr., Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. J. Exper. Med., 1930, 52, 561.
10. Bunim, J. J., and McEwen, C., The antistreptolysin titer in rheumatic fever, arthritis and other diseases. J. Clin. Invest., 1940, 19, 75.
11. Myers, W. K., Keefer, C. S., and Holmes, W. F., Jr., The resistance to fibrinolytic activity of the hemolytic streptococcus with special references to patients with rheumatic fever and rheumatoid (atrophic) arthritis. J. Clin. Invest., 1935, 14, 113.
12. McEwen, C., Bunim, J. J., and Alexander, R. C., Bacteriologic and immunologic studies in arthritis. II. Results of various immunologic tests in different forms of arthritis. J. Lab. and Clin. Med., 1935–36, 21, 465.
13. Perry, C. B., Streptococcal antifibrinolysin in rheumatoid arthritis and spondylitis anklylopoietica. Ann. of the Rheum. Dis., 1940–1941, 2, 147.
14. Ash, R., Nonspecific precipitins for pneumococcic fraction C in acute infections. J. Infect. Dis., 1933, 53, 89.
15. Abernethy, I. J., and Avery, O. T., The occurrence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patient's sera and the effect of calcium on the flocculation reaction with C polysaccharide of pneumococcus. J. Exper. Med., 1941, 73, 173.
16. Avery, O. T., and McCarty, M., Personal communication.
17. Perlman, E., Bullowa, J. G. M., and Goodkind, R., An immunological and electrophoretic comparison of the antibody to C polysaccharide and the C-reactive protein of acute phase serum. J. Exper. Med., 1943, 77, 97.

VARIATION IN PROTEIN AND POLYSACCHARIDE CONTENT OF SERA IN THE CHRONIC DISEASES, TUBERCULOSIS, SARCOIDOSIS, AND CARCINOMA [1]

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It is well-known that marked changes may occur in the blood proteins in many diseases, and these changes have usually been expressed as variations in the albumin to globulin (A/G) ratio. In 1937, Tiselius (1) showed by means of the electrophoresis technic that at least 3 distinctly different globulins can be distinguished in serum, and later work (2) made it possible to measure them quantitatively from their electrophoretic diagrams. Furthermore, it has been shown that the relative amounts of these different protein components vary considerably in different diseases. For example, various pathological sera and plasmas were studied with this technic by Longsworth, Shedlovsky and MacInnes (3); pneumonia sera, by Blix (4); sarcoidosis, by Fisher and Davis (5), and also by Seibert and Nelson (6); disseminated lupus erythematosus, by Coburn and Moore (7); multiple myeloma, by Kekwick (8), and also Gutman *et al* (9); tuberculosis, by Seibert and Nelson (6); malaria, by Dole and Kendall (10); rheumatic fever, by Dole, Watson and Rothbard (11), and also by Rutstein, Clarke and Taran (12), and many other diseases.

In certain cases, extra specific serum components (2, 13) with mobilities different from those of the normal globulins appear. Such components have frequently been shown to be antibodies. In most cases, however, increases in the components with normal globulin mobilities occur, indicating an increase in the amount of the normal proteins or the presence of an additional substance such as another protein, lipoid (14), carbohydrate (15, 14), etc., having a similar mobility as, or attached to the normal protein. Thus, apparently similar changes, and therefore similar patterns, may be seen in different diseases, reflecting similar physiological disturbances; but they may also, on the other hand, be due to specific causes. However, if sufficient other supporting data are available, the electrophoretic patterns may become very useful in confirming a diagnosis, and certainly are valuable in prognosis. For example, the present study shows that there can, in most cases, be a helpful differentiation between sera in cases of pulmonary tuberculosis, sarcoidosis, and carcinoma.

A number of studies have shown that an abnormal increase in lipoid occurs in the sera in certain diseases (3, 14), and this increase is seen in the α and β globulin components, since the lipoid has been found usually to accompany these fractions. Less attention has been paid to another important colloidal constituent of the serum; namely, polysaccharide. While its concentration is very much less than that of the serum proteins, it is present in as high or higher concentration than glucose, and may increase as much as 100 per cent or more in some cases. Lustig and Langer (16) found increases in tuberculosis, carcinoma and pneumonia. Nilsson (15) and Blix, Tiselius and Svensson (14) also found an increase in cases of pneumonia. Friedmann and Sutliff (17) noted the presence of a substance in the serum in certain diseases, probably a polysaccharide, from which the pneumococcus is capable of forming large quantities of acid.

A preliminary report was published (18) showing that a progressive increase in serum polysaccharide parallels advance in the tuberculous process. It seemed of interest, therefore, to correlate this change with the changes observed by electrophoresis in the different protein components, and thus to determine whether the increase in polysaccharide could account for any of these changes.

In this study, therefore, cases of relatively uncomplicated tuberculosis were carefully selected from the Henry Phipps Institute Clinic by one of

[1] Aided by a grant from the Committee on Medical Research of the National Tuberculosis Association, and also by chemical assistance from the Tuberculosis Control Division of the United States Public Health Service.

us on the basis of the clinical findings, and classified as to the stage of tuberculosis. Cases of Boeck's sarcoid which were strongly suspected on the basis of the clinical examinations, or proved by biopsy, were studied. Sera from patients with carcinoma were obtained, and also 6 cases each of diabetes and of diabetes complicated with tuberculosis. Normal adult and also fetal sera were studied. All these sera were analyzed for total protein, albumin, α_1, α_2, β and γ globulins, glucose, polysaccharide, and also sedimentation rate (except the diabetics and carcinomas, which were not secured in our clinic).

METHODS

Clinical appraisal

The terms minimal, moderately advanced, and far advanced pulmonary tuberculosis are used at the Henry Phipps Institute in accordance with the definitions of the National Tuberculosis Association (19). In addition each of these classes is subdivided into 3 groups: (*1*) active lesions for which treatment is indicated, (*2*) lesions of questionable clinical significance, and (*3*) lesions which are apparently healed and presumably without present clinical significance.

The term "active lesions" is used in this study to indicate lesions which in x-ray *appearance* are soft and flocculent, or have shown recent fluctuation, and disease from which tubercle bacilli have been recently demonstrated in the sputum.

"Lesions of questionable clinical significance" include infiltrations that have an x-ray appearance suggesting chronicity or stability, as indicated by fibrosis and scarring in part or parts of the lesion, with softer or less well defined shadows elsewhere suggesting that the disease is in part unhealed.

"Lesions which are apparently healed and presumably without clinical significance" include disease that appears to be wholly fibrotic or fibrocalcific, and disease that shows no evidence of fluctuation on serial x-ray examinations.

For use in the punch card system, these lesions are given the following code numbers (which are used in the following tables).

81 active minimal pulmonary tuberculosis.
82 active moderately advanced pulmonary tuberculosis.
83 active far advanced pulmonary tuberculosis.
84 minimal pulmonary tuberculosis of questionable clinical significance.
85 moderately advanced pulmonary tuberculosis of questionable clinical significance.

In the sarcoidosis series, 4 of the cases have been verified by study of biopsy material. The diagnoses of the others were based upon the presence of extensive pulmonary fibrosis, usually with enlargement of the lymph nodes at the hilum or mediastinum, and upon the absence of significant symptoms or deterioration of health, particularly in relation to the extent and density of the pulmonary shadows roentgenologically. In these cases, repeated examinations of the sputum were negative for tubercle bacilli, and the reactions to standard doses of Purified Protein Derivative were generally slight or absent. In 6 cases there were skin lesions typical of sarcoid lesions. Three of these were confirmed by consultation elsewhere. X-ray examinations of the bones of the hands and feet were not routinely made.

The diagnoses on the cases of carcinoma and diabetes were confirmed in each case in the respective institutions from which they were received. The carcinoma cases included lesions of the lungs, colon, rectum, stomach, pancreas, liver, brain, temple, tongue, breast, uterus and cervix.

Diabetic cases were studied in order to determine whether a change in the polysaccharide content of serum may be associated with the disturbed carbohydrate metabolism of this disease.

No attempt was made to obtain fasting bloods, since it had previously (20) been shown that the polysaccharide content of the serum is almost independent of the state of metabolism. The bloods were taken, allowed to clot, and within several hours the sera were removed and placed in a freezing box which kept them just at the point of freezing until the analyses could be made. It was shown (20) that under these conditions no change occurs in the polysaccharide or glucose content of serum over long periods of time.

Sedimentation. A small sample of the blood, immediately after being drawn, was mixed with oxalate and used for the sedimentation test, according to the method of Cutler (21), in the routine clinical laboratory of our institute.

Polysaccharide was determined by the carbazole reaction according to the procedure outlined recently (20), using the glucose-galactose-mannose standard. In this study, the total polysaccharide of serum was determined, and from it the glucose concentration, determined by the Somogyi (22) method, was subtracted, giving the true polysaccharide concentration.

Total protein was determined by the Kingsley (23) biuret method, using the Klett colorimeter. The calibration curve was made against serum protein determined by means of the micro Kjeldahl method.

Protein patterns were made by means of the Tiselius electrophoresis technic (1). Earlier studies (24, 6) using phosphate buffer, pH 7.7 $\mu = 0.1$, showed that a progressive rise in γ globulin paralleled the development of tuberculosis. In addition to this change, abnormalities were noted in the form of the albumin curve, of such a nature that often there seemed to be present a component with a slightly greater mobility than the albumin. This abnormality in the albumin component does not occur if the electrophoresis is made in barbiturate buffer, but instead an extra α globulin appears, as first shown by Longsworth (25). It is possible, therefore, that the extra α globulin resolved in this latter buffer may represent the same component included in the albumin fraction in the phosphate buffer.

All the experiments in the present study were, therefore, carried out in barbiturate buffer (veronal) pH 8.5 to 8.6, $\mu = 0.1$, after the serum was dialyzed, for several days against this buffer at 1° C. and then filtered and diluted 1:2 with the same buffer.

In all determinations the same buffer concentration and pH and also the same protein concentration were used, since variation in these conditions leads to variation in the percentage of the different components (26, 27). In these experiments the crossed-slit arrangement of Svensson (28) was used. All electrophoreses were continued for exactly 2 hours at about 8.1 to 8.8 volts per cm., during which time the components spread throughout most of the long type cell.

Earlier reports favored using only the descending pattern for measurements, since fewer boundary errors occurred on this side, as evidenced by the smaller ϵ peak. The ϵ peak represents a gradient due to buffer electrolyte alone, whereas the δ boundary on the ascending side is due to concentration gradients of both buffer salts and proteins.

These boundary anomalies, the δ and ϵ effects, can be greatly reduced by reducing protein concentration and also by increasing the ionic strength of the buffer (29, 26, 27). In fact, they can be so much reduced, when a total ionic strength of 0.2 to 0.25 is used, that Svensson now recommends using the patterns on both sides, and taking an average of the results for the corresponding components on the 2 sides.

However, in the present study, since an ionic strength of 0.1 was used, calculations were made only from the descending patterns. Since relative rather than absolute values are desired, any error due to this method of calculation would have no significant bearing on the conclusions drawn. No difficulties were encountered with β anomalies so frequently reported by many workers.

Diagrams were enlarged 5 times, and the areas due to the different components were always resolved by the same individual into curves, as described by Pedersen (30), and measured with a planimeter. Since the components are not always definitely resolved into isolated peaks, a certain amount of personal error enters into these resolutions, and Table I will show the degree of error involved under the conditions described. In some cases, the same serum was examined on 2 successive days, and in others on widely different days, even as much as 3 months apart. Different exposures during the same experiment were also compared, using different angles on the diagonal slit, in order to obtain patterns of different sizes. No systematic error seemed to be caused by such variation, but nevertheless the same angle, 30°, was used in all experiments in the comparative series reported in this paper. In general, as much as 2 per cent error may be expected in the globulin components, and 3 per cent in the albumin, under these conditions.

Results are reported as percentage of the total gradient area (exclusive of the ϵ component), as the different components, albumin, α_1, α_2, β, and γ globulins. This method of calculation involves the least error, since no committal is made as to the composition of these components. It is known that a considerable percentage of some of these components is not protein. For example, it was emphasized earlier that considerable lipoid and carbohydrate accompany the α and β components. However, even these results do not give an entirely true picture for comparison, since the total protein and colloidal concentration of different sera vary. Therefore, in spite of the obvious errors involved, the method of calculating the actual grams per cent of each component from the total protein concentration, as used extensively by other workers (31, 32), is also used here, and the results are recorded as the second item under each component. While the actual content in grams of each component could also be calculated directly from the electrophoretic diagrams, no more accurate results would be obtained, since the refractive index increments of the various colloidal constituents necessary in the calculations are also not known.

In spite of the inherent errors indicated, the differences recorded for the different stages of disease and different diseases are so great that the conclusions drawn are, without doubt, valid.

TABLE I

Electrophoretic measurements of same serum at different times, and with different angles at same time in veronal buffer pH 8.5 to 8.7, $\mu = 0.1$

Name	Date bled	Date run	Percentage of total protein as					Angle
			Alb.	α_1	α_2	β	γ	
S.F.	1-16-45	1-23-45	40.1	10.8	13.7	12.7	22.8	30°
		4- 5-45	41.1	10.2	14.7	12.2	21.9	30°
M.G.	8-17-45	8-25-45	29.1	10.9	20.6	21.5	18.0	45°
		11- 8-45	28.9	12.2	20.0	21.9	17.1	45°
			29.3	11.0	20.1	22.2	17.5	55°
S.F.	2- 2-45	2-19-45	53.6	7.5	11.7	12.3	14.9	30°
		4-20-45	53.4	8.0	11.8	12.6	14.2	30°
			52.2	7.7	11.6	13.4	15.1	25°
			50.2	8.6	11.9	13.4	15.9	20°
F.J.	3- 7-45	3-23-45	50.0	8.4	17.6	12.9	11.1	30°
		4-25-45	51.2	7.1	18.0	11.3	12.5	30°
M.C.	2-20-45	4- 3-45	49.1	7.5	10.3	19.1	14.1	30°
		4- 4-45	50.6	8.0	9.8	17.5	14.1	30°
C.R.	2- 8-45	3-12-45	55.2	7.3	9.5	11.6	16.4	30°
		4-27-45	52.6	9.1	9.6	11.8	16.9	30°
A.F.	2- 7-45	3-14-45	45.9	9.9	12.0	15.9	16.4	30°
		4- 5-45	46.8	8.7	12.3	15.4	16.8	30°
J.B.	2-10-45	3-27-45	50.6	8.8	14.7	13.7	12.2	35°
		3-29-45	50.0	8.7	14.7	14.1	12.4	35°
S.S.	2- 5-45	3- 7-45	52.4	8.2	11.1	14.7	13.7	30°
			51.1	8.8	11.6	14.1	14.3	35°
J.G.	1-10-44	2- 3-44	44.6	6.8	12.2	17.2	19.4	35°
			41.1	7.8	12.7	18.4	19.9	45°
S.C.	7- 9-46	7-16-46	34.3	10.9	16.3	14.7	23.9	30°
			33.4	11.5	16.1	13.7	25.3	30°

RESULTS

Since it is impossible to include the actual analyses of all cases, because of the large number, an attempt has been made to give summarizing tables which will contain data to show for each type of case the mean, the standard deviation, the probable error, the range, and also the significance of the deviations from the normal means.

The method of calculating this significance is as follows.

The standard deviation $(\sigma) = \sqrt{\frac{\Sigma d^2}{N}}$, where d = the difference from the mean, and N = the number of cases studied.

Probable error $(E) = 0.6745 \frac{\sigma}{\sqrt{N}}$.

$a_1 - a_2$ = the difference between the mean (a_1) of the normal group and that of the group under study (a_2).

Probable error of $(a_1 - a_2) = \sqrt{E_1^2 + E^2}$.

The significance of the differences from the normal mean is shown by the ratio

$$\frac{a_1 - a_2}{\sqrt{E_1^2 + E_2^2}}.$$

For example, if this ratio is 5, then, according to Gavett (33), the odds against getting a mean of a similar group which is outside of the probable error are about 1,300 to 1, and the difference is therefore highly significant. If the ratio is 3 the odds are only 22 to 1; if the ratio is 4 the odds are 142 to 1; if it is 6 the odds are 20,000 to 1; if it is 7 the odds are 445,000 to 1; etc.

Table II shows, in the top half, the number of cases studied and their means for the different analyses. The lower half gives the ratios, which show the significance of the deviations of these means from the normal. The electrophoretic data

TABLE II

Means and their significance

Diagnosis	Number cases	Polysaccharide	Total protein	Albumin		Globulins								Albumin to globulin ratio (A/G)
						α_1		α_2		β		γ		
		mgm. per cent	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	
Means														
Normal adult	43	103	7.29	53.3	3.88	8.0	0.58	10.4	0.76	13.8	1.01	14.2	1.05	1.15
Fetal	6	69	6.13											
Tuberculosis 81	20	109	7.27	49.8	3.62	7.7	0.56	10.8	0.78	13.8	1.00	17.8	1.30	1.00
82	22	136	7.67	43.5	3.32	8.5	0.64	12.2	0.92	14.8	1.15	21.2	1.65	0.83
83	26	159	7.63	35.2	2.69	10.3	0.78	16.1	1.21	15.6	1.19	23.0	1.76	0.56
84	21	109	7.17	51.7	3.71	8.2	0.59	11.4	0.81	13.9	1.00	14.8	1.06	1.08
85	13	112	7.20	49.4	3.54	8.6	0.62	11.6	0.84	15.5	1.11	14.9	1.08	0.99
Sarcoidosis	11	128	7.88	39.4	3.10	7.8	0.61	10.8	0.85	15.9	1.26	26.1	2.06	0.66
Carcinoma	23	145	6.66	42.0	2.80	10.0	0.66	16.5	1.09	16.3	1.09	15.3	1.03	0.74
Diabetes	6	115	7.16	51.1	3.65	6.9	0.50	11.3	0.81	17.1	1.23	13.6	0.98	1.05
Diabetes + tuberculosis	6	166	6.82	30.4	2.11	10.9	0.74	15.2	1.03	19.5	1.33	24.0	1.62	0.46
$a_1 - a_2/\sqrt{E_1^2 + E_2^2}$ (Significance of variations from the means)														
Normal adult	43	σ												
Fetal	6	9.7	9.5											
Tuberculosis 81	20	2.5	0.2	5.2	4.3	1.1	1.2	1.5	1.0	0	0.5	5.5	4.3	5.2
82	22	10.1	4.3	9.2	8.7	1.6	3.0	5.3	8.0	2.1	3.5	7.1	6.5	10.0
83	26	19.1	3.5	24.4	19.8	7.9	11.7	13.6	18.0	5.4	6.0	10.8	8.9	29.5
84	21	2.0	1.5	3.1	2.8	0.8	0.5	2.9	2.5	0.3	0.5	1.3	0.25	3.5
85	13	2.1	1.1	7.5	6.8	2.9	2.6	2.1	1.6	3.8	3.3	1.1	0.6	8.0
Sarcoidosis	11	6.3	7.4	13.5	8.7	0.7	1.5	0.95	2.3	5.6	8.3	7.9	10.1	12.3
Carcinoma	23	10.6	5.2	13.0	15.0	6.7	4.0	12.7	11.0	6.4	2.7	1.8	0.5	20.5
Diabetes	6	2.1	1.9	2.5	4.6	6.5	4.0	2.3	2.5	6.2	5.5	0.63	1.4	3.3
Diabetes + tuberculosis	6	4.9	2.6	9.5	8.4	5.0	5.3	8.0	6.8	10.2	8.0	5.2	5.7	13.8

were calculated both on the basis of percentage of the total, and also as the actual grams per cent, assuming that all of the components are protein, or that the error due to the presence of non-protein substances is negligible.

Table III contains the standard deviations and probable errors corresponding to the results in Table II. Table IV contains the data,

$$a_1 - a_2 \pm \sqrt{E_1^2 + E_2^2},$$

used for calculating the significance ratios, and also the ranges of the different analyses, for use in deciding what to expect from analyses on individual sera.

From these results it is readily seen that there is a significant increase in the mean total protein concentration in sarcoidosis, and a significant decrease in carcinoma, while in tuberculosis, regardless of the stage of the disease, neither an increase nor decrease is the rule.

Figure 1 shows typical electrophoretic diagrams of each type and stage of disease studied; the individual patterns were chosen because their analyses fitted most closely to the mean values for each particular type of case.

Figures 2, 3 and 4 show the individual polysaccharide concentrations plotted respectively against the α_2 globulin, γ globulin, and albumin, since it was in these components that there proved to be significant variations. In all cases the dots represent normal sera, the triangles minimal, the circles moderately advanced, the black squares far advanced tuberculosis, and the crosses carcinoma.

In minimal active tuberculosis (tuberculosis 81) a decrease in the mean A/G ratio is significant, and this is obviously due to the increase in the γ globulin, with a corresponding decrease in albumin. Therefore, the most significant change found in this early stage of the disease is the rise in the γ globulin. As the disease progresses to the mod-

TABLE III

Standard deviations and probable errors

Diagnosis	Polysaccharide	Total protein	Albumin		Globulins α_1		Globulins α_2		Globulins β		Globulins γ		A/G ratio
	mgm. per cent	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	
Standard deviations													
Normal adult	14.0	0.35	2.6	0.23	1.35	0.10	1.38	0.10	1.89	0.14	2.68	0.22	0.12
Fetal	11.6	0.42											
Tuberculosis 81	13.2	0.59	4.1	0.37	1.45	0.09	1.49	0.11	1.42	0.12	3.6	0.35	0.18
82	20.9	0.56	7.2	0.44	1.90	0.13	2.23	0.15	3.08	0.28	6.7	0.62	0.19
83	18.9	0.67	5.2	0.44	2.00	0.11	3.02	0.18	2.10	0.19	5.8	0.60	0.12
84	16.9	0.48	3.1	0.34	1.40	0.10	2.10	0.14	2.10	0.15	2.4	0.21	0.14
85	20.1	0.44	3.4	0.26	0.87	0.06	2.90	0.25	2.20	0.14	3.0	0.25	0.12
Sarcoidosis	17.9	0.36	4.9	0.47	1.20	0.09	2.00	0.16	1.70	0.15	7.7	0.63	0.19
Carcinoma	25.9	0.78	5.9	0.53	1.90	0.13	3.30	0.20	2.50	0.23	3.7	0.32	0.17
Diabetes	18.5	0.23	3.0	0.17	0.86	0.06	1.30	0.08	1.80	0.14	3.4	0.19	0.12
Diabetes + tuberculosis	45.8	0.64	8.5	0.73	2.04	0.09	2.10	0.13	1.90	0.15	5.7	0.34	0.17
Probable errors of the means													
Normal adult	1.43	0.036	0.27	0.024	0.14	0.01	0.14	0.01	0.19	0.01	0.27	0.023	0.012
Fetal	3.20	0.117											
Tuberculosis 81	1.98	0.089	0.61	0.056	0.22	0.014	0.22	0.017	0.21	0.018	0.59	0.053	0.03
82	3.01	0.080	1.03	0.060	0.27	0.02	0.31	0.02	0.44	0.04	0.96	0.09	0.03
83	2.50	0.090	0.69	0.060	0.26	0.014	0.40	0.02	0.28	0.03	0.77	0.08	0.02
84	2.50	0.070	0.45	0.050	0.21	0.02	0.31	0.02	0.31	0.02	0.35	0.03	0.02
85	3.75	0.080	0.45	0.050	0.16	0.011	0.56	0.05	0.41	0.03	0.56	0.05	0.02
Sarcoidosis	3.70	0.070	1.00	0.090	0.26	0.02	0.40	0.04	0.32	0.03	1.50	0.10	0.04
Carcinoma	3.60	0.110	0.83	0.070	0.27	0.02	0.46	0.03	0.35	0.03	0.52	0.04	0.02
Diabetes	5.20	0.060	0.84	0.050	0.24	0.02	0.37	0.02	0.50	0.04	0.91	0.05	0.03
Diabetes + tuberculosis	12.80	0.180	2.40	0.210	0.57	0.03	0.59	0.04	0.53	0.04	1.60	0.10	0.05

TABLE IV

Ranges and significance data

Diagnosis	Polysaccharide	Total protein	Albumin		Globulins								A/G ratio
					α_1		α_2		β		γ		
	mgm. per cent	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	*per cent*	*grams per cent*	
Ranges													
Normal adult	73 to 131	6.3 to 8.2	47.6 to 59.2	3.4 to 4.3	5.3 to 11.5	0.38 to 0.87	7.7 to 12.9	0.57 to 0.94	7.9 to 17.6	0.73 to 1.38	8.7 to 19.7	0.59 to 1.16	0.91 to 1.45
Fetal	46 to 81	5.4 to 6.6											
Tuberculosis 81	83 to 133	6.2 to 8.2	44.0 to 58.4	2.8 to 4.2	4.5 to 10.0	0.32 to 0.74	8.1 to 14.2	0.58 to 0.93	11.6 to 17.5	0.82 to 1.27	10.5 to 25.1	0.73 to 1.91	0.78 to 1.40
82	102 to 191	6.9 to 8.8	26.7 to 52.8	2.2 to 3.9	5.4 to 13.3	0.46 to 0.93	9.0 to 17.7	0.73 to 1.32	11.1 to 21.4	0.76 to 2.00	12.7 to 31.1	0.98 to 2.88	0.36 to 1.12
83	120 to 205	5.9 to 9.0	27.0 to 42.2	1.6 to 3.4	7.5 to 15.8	0.59 to 0.94	10.8 to 22.7	0.88 to 1.58	11.2 to 22.1	0.79 to 1.66	14.2 to 36.5	0.97 to 3.28	0.37 to 0.77
84	70 to 140	6.4 to 8.2	45.8 to 58.5	3.2 to 4.4	5.3 to 11.6	0.34 to 0.93	8.8 to 17.5	0.60 to 1.23	11.0 to 18.0	0.74 to 1.33	10.3 to 19.8	0.74 to 1.92	0.85 to 1.41
85	66 to 149	6.3 to 8.4	39.3 to 52.3	2.9 to 4.0	7.6 to 10.7	0.52 to 0.70	8.1 to 18.9	0.55 to 1.54	8.7 to 17.6	0.71 to 1.25	9.7 to 20.9	0.61 to 1.56	0.65 to 1.10
Sarcoidosis	102 to 170	7.3 to 8.3	27.3 to 49.0	2.1 to 3.8	6.0 to 9.6	0.45 to 0.76	6.4 to 13.3	0.52 to 1.08	11.9 to 18.6	0.91 to 1.51	16.1 to 37.4	1.18 to 2.88	0.38 to 0.96
Carcinoma	91 to 197	5.4 to 8.1	26.5 to 52.0	1.6 to 3.8	7.4 to 13.9	0.46 to 0.92	10.6 to 23.7	0.72 to 1.56	11.2 to 23.0	0.74 to 1.87	8.1 to 21.7	0.46 to 1.63	0.36 to 1.08
Diabetes	92 to 147	6.9 to 7.5	45.3 to 54.8	3.4 to 3.8	5.8 to 8.4	0.40 to 0.58	9.5 to 13.4	0.70 to 1.00	14.0 to 19.7	0.96 to 1.44	11.9 to 19.7	0.73 to 1.26	0.83 to 1.21
Diabetes + tuberculosis	98 to 245	6.2 to 8.1	17.5 to 39.4	1.1 to 3.1	7.5 to 13.0	0.61 to 0.83	12.1 to 18.1	0.81 to 1.27	16.5 to 22.9	1.15 to 1.54	16.2 to 31.3	1.06 to 2.17	0.21 to 0.65
$a_1 - a_2 \pm \sqrt{E_1^2 + E_2^2}$													
Normal adult													
Fetal	34.5 ± 3.56												
Tuberculosis 81	6.0 ± 2.44	0.02 ±0.01	3.5 ±0.67	0.26 ±0.06	0.3 ±0.26	0.02 ±0.02	0.4 ±0.26	0.02 ±0.02	0 ±0.28	0.01 ±0.02	3.6 ±0.65	0.25 ±0.06	0.15 ±0.03
82	33.4 ± 3.3	0.38 ±0.09	9.8 ±1.06	0.56 ±0.06	0.5 ±0.30	0.06 ±0.02	1.8 ±0.34	0.16 ±0.02	1.0 ±0.47	0.14 ±0.01	7.0 ±0.99	0.60 ±0.09	0.32 ±0.03
83	55.5 ± 2.9	0.34 ±0.09	18.1 ±0.74	1.19 ±0.06	2.3 ±0.29	0.20 ±0.02	5.7 ±0.42	0.45 ±0.03	1.8 ±0.03	0.18 ±0.03	8.8 ±0.81	0.71 ±0.08	0.59 ±0.02
84	5.8 ± 2.9	0.12 ±0.08	1.6 ±0.52	0.17 ±0.06	0.2 ±0.25	0.01 ±0.02	1.0 ±0.34	0.05 ±0.02	0.1 ±0.36	0.01 ±0.02	0.6 ±0.44	0.01 ±0.04	0.07 ±0.02
85	8.6 ± 4.0	0.09 ±0.08	3.9 ±0.52	0.34 ±0.05	0.6 ±0.21	0.04 ±0.02	1.2 ±0.58	0.08 ±0.05	1.7 ±0.45	0.10 ±0.03	0.7 ±0.62	0.03 ±0.05	0.16 ±0.02
Sarcoidosis	24.9 ± 4.0	0.59 ±0.08	13.9 ±1.03	0.78 ±0.09	0.2 ±0.29	0.03 ±0.02	0.4 ±0.42	0.09 ±0.04	2.1 ±0.37	0.25 ±0.03	11.9 ±1.52	1.01 ±0.10	0.49 ±0.01
Carcinoma	41.5 ± 3.9	0.63 ±0.12	11.3 ±0.87	1.08 ±0.07	2.0 ±0.30	0.08 ±0.02	6.1 ±0.48	0.33 ±0.03	2.5 ±0.39	0.08 ±0.03	1.1 ±0.59	0.02 ±0.04	0.11 ±0.02
Diabetes	11.6 ± 5.4	0.13 ±0.07	2.2 ±0.88	0.23 ±0.05	1.9 ±0.24	0.08 ±0.02	0.9 ±0.39	0.05 ±0.02	3.3 ±0.53	0.22 ±0.04	0.6 ±0.95	0.07 ±0.05	0.10 ±0.03
Diabetes + tuberculosis	62.6 ± 12.80	0.47 ±0.18	22.9 ±2.4	1.77 ±0.21	2.9 ±0.58	0.16 ±0.03	4.8 ±0.60	0.27 ±0.01	5.7 ±0.56	0.32 ±0.01	9.8 ±1.9	0.57 ±0.10	0.69 ±0.05

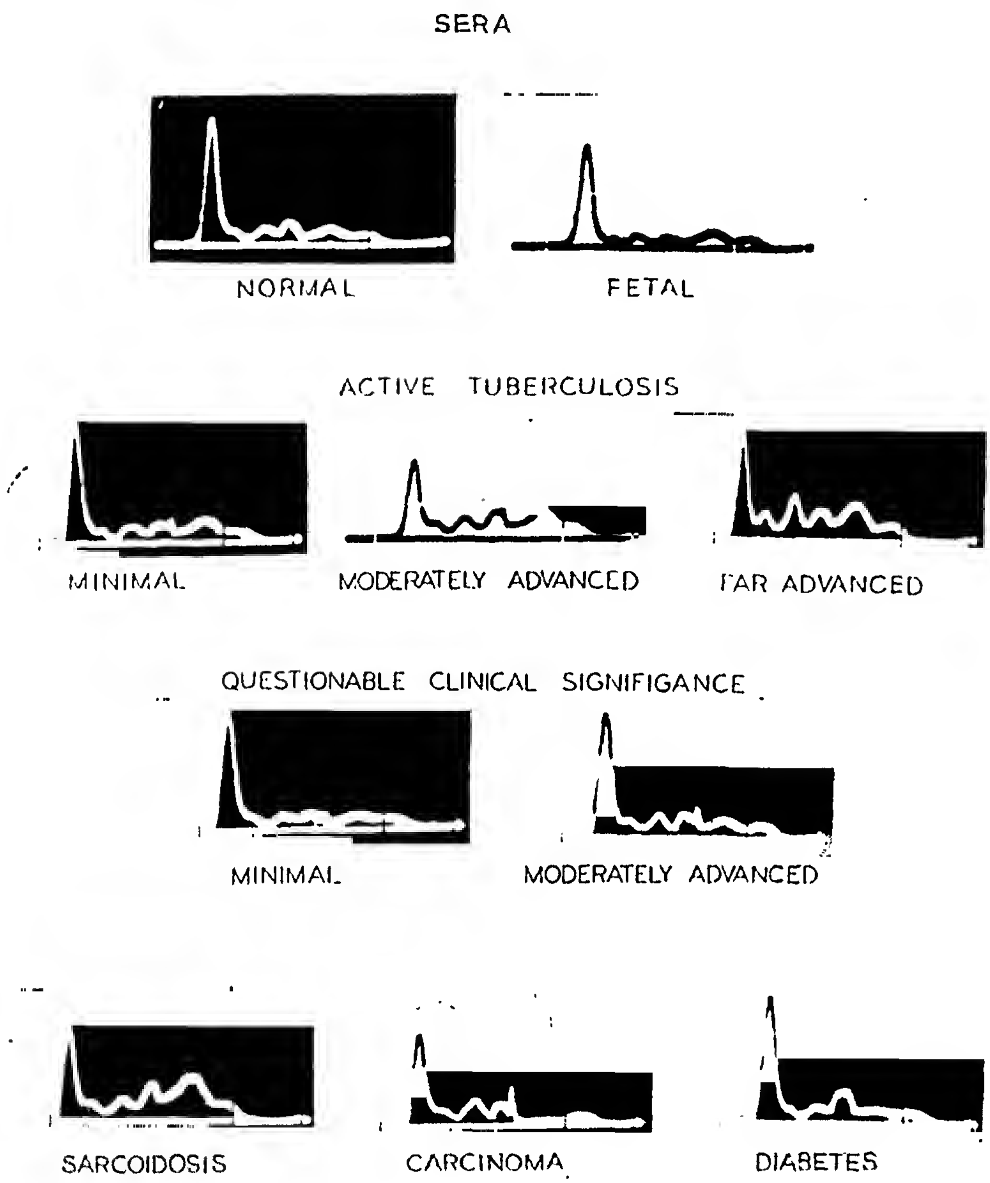

FIG. 1. ELECTROPHORETIC DIAGRAMS OF SERA EXAMINED IN VERONAL BUFFER pH 8.6, $\mu = 0.1$, AND AT A 30° ANGLE

Only descending patterns are shown. In all cases the albumin, α_1, α_2, β and γ globulin peaks and the ϵ boundary are evident.

erately advanced stage (tuberculosis 82), there appeared also a rise in the α_2 globulin and polysaccharide content. With far advanced disease (tuberculosis 83) all globulins, and especially α_2, as well as polysaccharide content, increased with a corresponding decrease in albumin, even though ıe total protein changed only slightly.

ı those cases of minimal tuberculosis classified as of questionable clinical significance (tuberculosis 84), no deviation from the normal was found.

In cases of moderately advanced tuberculosis of questionable clinical significance (tuberculosis 85), the only significant change is a decrease in the albumin and the corresponding lowered A/G ratio.

In sarcoidosis, a large increase occurred in the mean γ globulin, and also in the β globulin, a

simultaneous decrease in albumin and consequently in the A/G ratio. There was also some increase in polysaccharide but by no means as much as found in cases of far advanced tuberculosis, which showed similar increases in the γ globulin. The total protein also was always increased.

In only 4 of the 11 cases reported in this study was the diagnosis proved by biopsy. The others were strongly suspected to be sarcoidosis from the clinical data. While the number of cases is comparatively few, it is believed the conclusions are valid, since a previous group of sarcoid cases (6) yielded comparable results, and also Fisher and Davis (5) obtained similar results on a group of 12 cases of sarcoid, all of which were proved by biopsy.

Carcinoma sera showed, on the other hand, no increase in the mean γ globulin. In fact, in the majority of cases, a very much flattened γ peak (see Figure 1) occurred, which in further studies may be possibly resolved into a very small true γ globulin, and another small component with mobility between the γ and β globulins. Several sera, especially from those cases with metastases to the liver and a resulting jaundice, showed an abnormally high γ globulin, and these brought the average γ up to near the normal mean. The α_2 globulin, and also the polysaccharide content, in practically all cases were conspicuously increased and, of course, the albumin and A/G ratio were decreased. The total protein in most cases was reduced.

The series of diabetic cases is admittedly too small to permit reliable conclusions, but it is obvious that no great change occurred, except possibly in the β globulin. Since 5 out of the 6 cases showed a marked increase in this component, and since fat metabolism is known to be disturbed in diabetes, further study of this disease may yield helpful results.

The diabetics with advanced tuberculosis showed

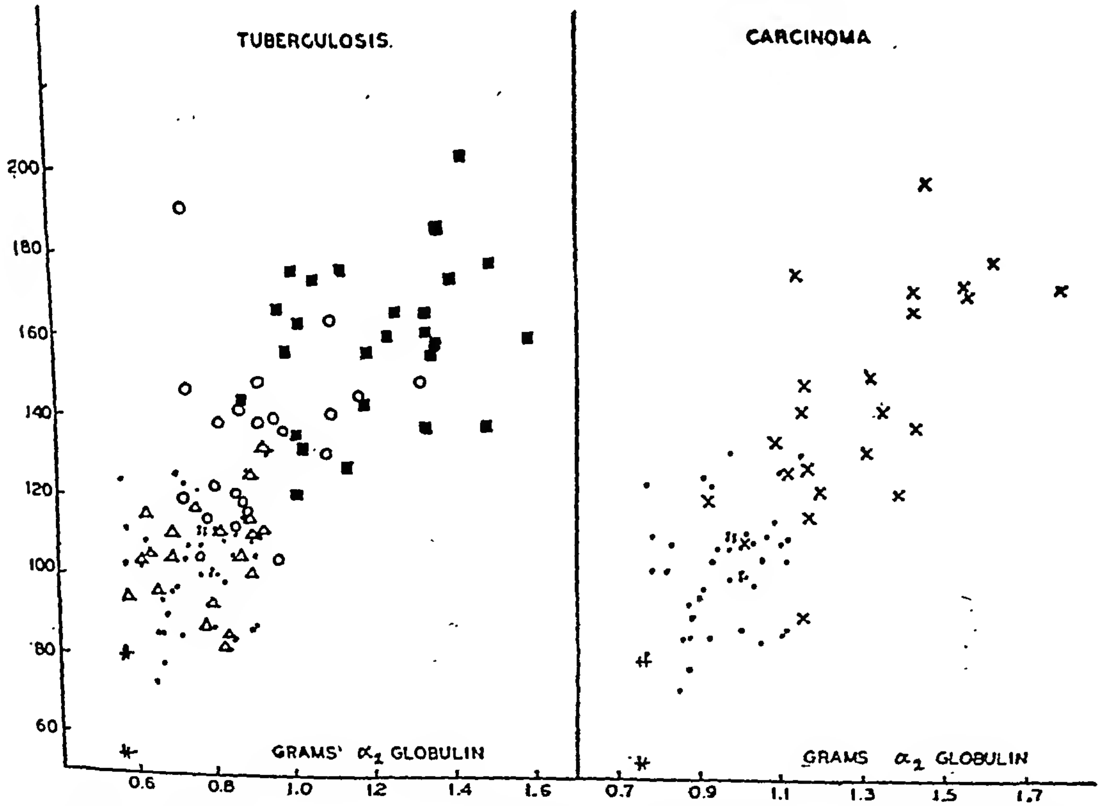

FIG. 2. GRAMS PER CENT α_2 GLOBULIN ARE PLOTTED AGAINST MGM. PER CENT POLYSACCHARIDE FOR NORMAL ADULT (•), FETAL (ɫɫ), MINIMAL (Δ), MODERATELY ADVANCED (O) AND FAR ADVANCED TUBERCULOSIS (■), AND CARCINOMA (X)

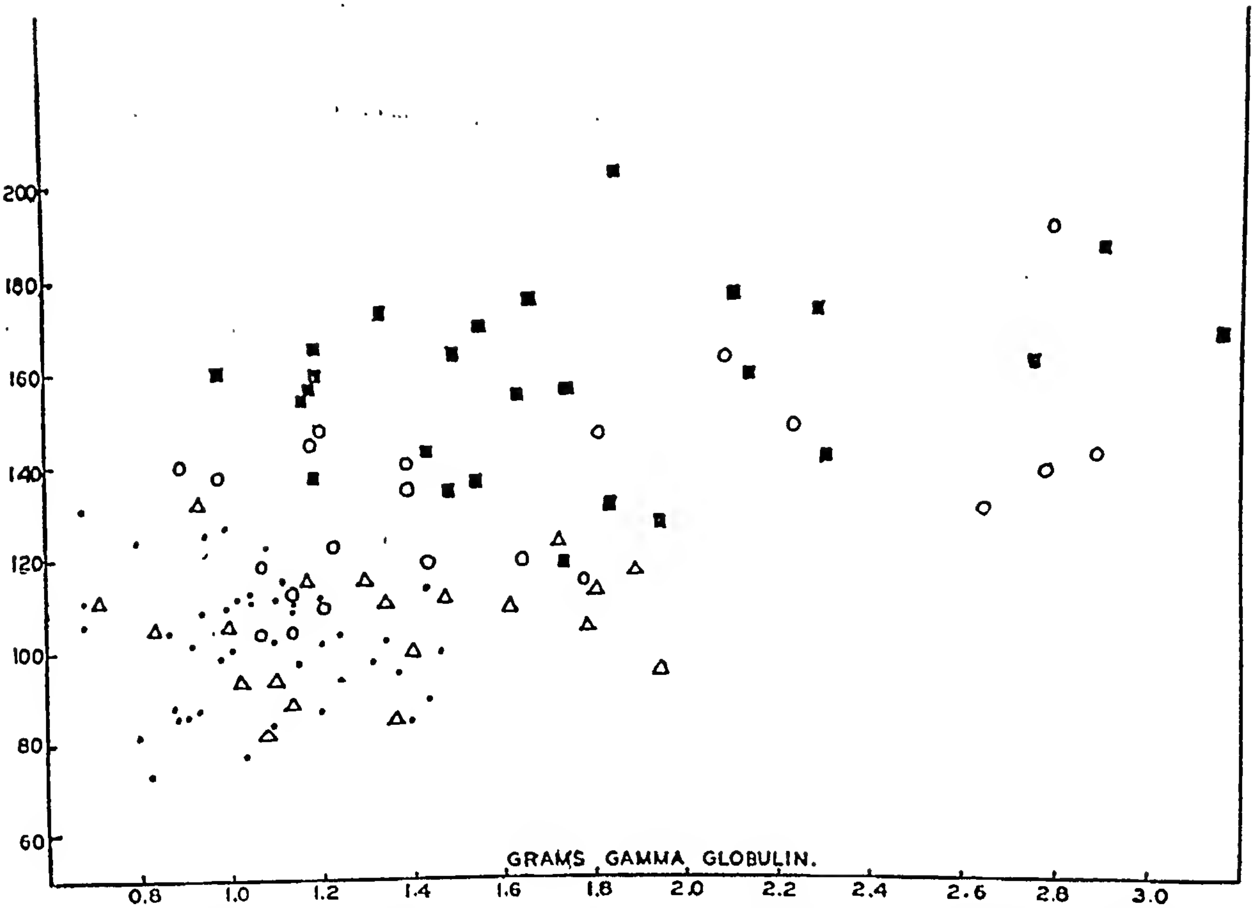

FIG. 3. GRAMS PER CENT γ GLOBULIN ARE PLOTTED AGAINST MGM. PER CENT POLYSACCHARIE FOR NORMAL ADULT (•), MINIMAL (△), MODERATELY ADVANCED (O) AND FAR ADVANCED TUBERCULOSIS (■)

changes similar to those found in the tuberculous patients, except possibly a still higher β globulin, thus adding significance to the increase in this component noted in the non-tuberculous diabetics.

Polysaccharide content of fetal serum.

Sera taken from the cord blood of 6 patients at parturition showed a very much lowered content of polysaccharide. Even if the lowered total protein content should be taken as an index of a dilution factor, still the corrected polysaccharide content would correspond only to the lowest figures obtained in a few adults in the normal group. There is an indication, therefore, that this constituent tends to increase with age.

Only 2 electrophorectic analyses were made of fetal sera, since the results obtained corresponded well with those already reported (32). The absolute and relative concentrations of γ globulin are higher than the adult values, and the other globulin concentrations are considerably lower, including especially the α_2 globulin.

Sedimentation test

In general, with far advanced tuberculosis, the sedimentation rate was abnormal and the serum constituents were also found to be abnormal; but with less advanced disease (tuberculosis 82), 6 cases out of 20 were found to have a normal sedimentation rate, while at the same time their sera showed a considerable increase over the mean of γ globulin and in some cases of polysaccharide. Among the group of 43 normal adults in whom no obvious clinical abnormality could be detected, 9 had sedimentation rates of 2 or more, and in all of these one of the globulins or polysaccharide, or both, was among the high range of normals. There were also 3 who had completely normal sedimentation rates, but had the highest values for polysaccharide (131, 124, 126 mgm. per cent) which were included among our normal group.

In view of the many factors which can influence the sedimentation rate, no definite correlations can be drawn on the basis of the present work.

They do not, however, conflict with the conclusion drawn by Shedlovsky (37) that there is a correlation between the increase in α globulin and increase in sedimentation rate, except in our group of sarcoids where 6 cases showed an increased sedimentation rate, but no increase in α globulin.

CONCLUSIONS AND DISCUSSION

The data show a slight but significant rise in the mean γ globulin component in early tuberculosis. Since antibodies for most diseases have been found in the γ globulin fraction (34), and, furthermore, since antibodies in tuberculosis have also been found in this fraction (35, 36), it is reasonable to suspect that the rise in this component in the early stage of the disease process may represent an antibody reaction to the organism. It will be of interest to determine whether the rise in this component has any prognostic value in differentiating potentially dangerous cases from the self-healing type. Such a study is in progress.

As the disease progresses, the next change to appear is an increase in the α_2 globulin and polysaccharide content of sera, and finally with far advanced disease, all of the globulins, and especially the α_2 globulin, increase, with a balancing decrease in the albumin, since the total protein content does not notably change. The polysaccharide also conspicuously increases. Therefore, with advance in the disease process, it appears that the accompanying tissue destruction may be reflected by this increase in the α_2 globulin and polysaccharide. It has been stated by Longsworth, Shedlovsky and MacInnes (3) that a rise in α globulin is found in febrile diseases. Shedlovsky and Scudder (37) concluded that a rise in α globulin indicated tissue destruction, and Perlmann, Glenn, and Kaufman (38) found an increase in the α globulin component of serum and of lymph in burns.

The argument that an increase in polysaccharide, as well as in α_2 globulin, indicates the presence of tissue destruction is supported by the

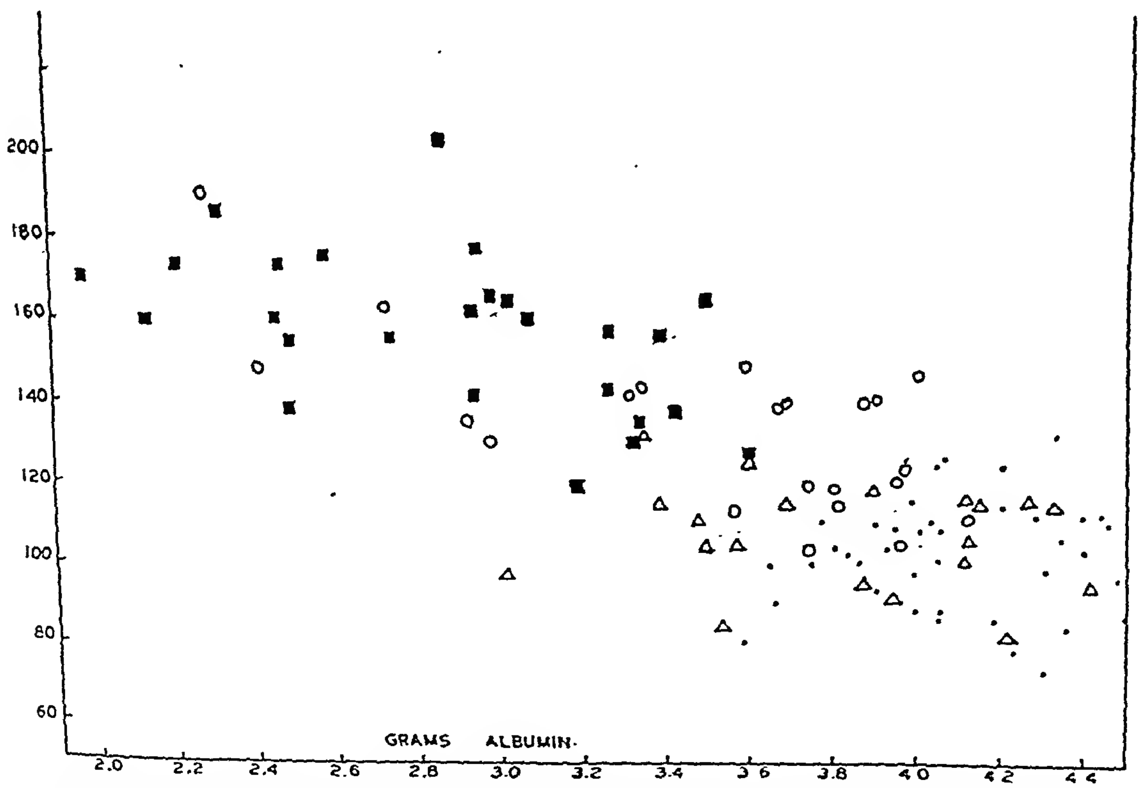

FIG. 4. GRAMS PER CENT ALBUMIN ARE PLOTTED AGAINST MGM. PER CENT POLYSACCHARIDE FOR NORMAL ADULT (•), MINIMAL (△), MODERATELY ADVANCED (O) AND FAR ADVANCED TUBERCULOSIS (■)

following facts. Isolated α_2 globulin contains a higher percentage of polysaccharide than do the other normal serum proteins (these results will be reported in a later communication), and these 2 constituents of serum always simultaneously increase. They are both low in fetal sera and, furthermore, increase in tuberculosis as well as in carcinoma, diseases where there is known to be tissue destruction, whereas in sarcoidosis, without tissue destruction, no comparable rise in α_2 globulin occurs, and, also, the rise in polysaccharide is much less. In this latter case the increase that is found in the polysaccharide is probably due to the rise in γ globulin which also does contain some polysaccharide, but possibly of a different nature.

As cases of tuberculosis tend to heal and are then viewed as of questionable clinical significance, there is a return to the normal serum picture. If the disease has been advanced, then a decrease in the albumin still persists. In other words, the albumin is the last component to return to normal, and this is especially significant in view of the fact that Zeldis and Alling (39) found the restoration of albumin to be slower than that of the other serum proteins following acute depletion by plasmapheresis.

It is of considerable importance that 3 diseases, which frequently are difficult to differentiate clinically or from the x-ray picture, namely, pulmonary tuberculosis, sarcoidosis, and carcinoma, present such different analytical pictures as here described. Such analyses could, therefore, be of considerable help in diagnosis.

SUMMARY

A statistically significant rise in the γ globulin occurs in the serum in minimal active tuberculosis, with a corresponding decrease in albumin. In moderately advanced disease, the α_2 globulin and polysaccharide content also increase. These changes are still further emphasized in far advanced tuberculosis, and all the globulins are also increased. The mean total protein, however, does not differ from the normal.

No deviation from the normal occurs in minimal tuberculosis of questionable clinical significance, except possibly in a slightly lowered A/G ratio. Moderately advanced tuberculosis of questionable clinical significance shows only a decrease in albumin, indicating that it is the last component to return to normal.

Sera from cases of sarcoidosis show an increase in total protein, a proportionately large increase in γ globulin, and only a moderate increase in polysaccharide content.

In carcinoma there is a decrease in total protein, a large increase in α_2 globulin and polysaccharide content.

Fetal sera show a low total protein and polysaccharide content and low globulins, except γ globulin, which is high.

It is suggested that the increase in γ globulin in minimal tuberculosis may indicate antibody formation, whereas the rise in α_2 globulin and simultaneous rise in polysaccharide in advanced tuberculosis and carcinoma may represent tissue destruction. Fetal sera as well as sera in sarcoidosis are characterized by high γ globulin.

Of protein fractions isolated from normal plasma, the α_2 globulin has the highest polysaccharide content. The increases in this component in the diseases studied apparently account for most of the increase in the polysaccharide of the sera. Some increase may be due also to the increase in γ globulin, as in sarcoidosis.

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BIBLIOGRAPHY

1. Tiselius, Arne, Electrophoresis of serum globulin. II. Electrophoretic analysis of normal and immune sera. Biochem. J., 1937, 31, 1464.

2. Tiselius, Arne, and Kabat, E. A., An electrophoretic study of immune sera and purified antibody preparations. J. Exper. Med., 1939, 69, 119.
3. Longsworth, Lewis G., Shedlovsky, T., and MacInnes, D. A., Electrophoretic patterns of normal and pathological human blood serum and plasma. J. Exper. Med., 1939, 70, 399.
4. Blix, Gunnar, Quantitative bestimmung von electrophoretisch getrennten serum globulin. Zeits. für ges. Exper. Med., 1939, 105, 595.
5. Fisher, A. M., and Davis, B. D., The serum proteins in sarcoid: electrophoretic studies. Bull. Johns Hopkins Hosp., 1942, 71, 364.
6. Seibert, F. B., and Nelson, J. W., Electrophoresis of serum. Serum proteins in tuberculosis and other chronic diseases. Am. Rev. Tuberc., 1943, 47, 66.
7. Coburn, A. F., and Moore, D. H., The plasma proteins in disseminated lupus erythematosus. Bull. Johns Hopkins Hosp., 1943, 73, 196.
8. Kekwick, R. A., The serum proteins in multiple myelomatosis. Biochem. J., 1940, 34, 1248.
9. Gutman, A. B., Moore, D. H., Gutman, E. B., McClellan, V., and Kabat, E. A., Fractionation of serum proteins in hyperproteinemia with special reference to multiple myeloma. J. Clin. Invest., 1941, 20, 765.
10. Dole, Vincent P., and Emerson, K., Jr., Electrophoretic changes in the plasma protein patterns of patients with relapsing malaria. J. Clin. Invest., 1945, 24, 644.
11. Dole, Vincent P., Watson, R. F., and Rothbard, S., Electrophoretic changes in the serum protein patterns of patients with scarlet fever and rheumatic fever. J. Clin. Invest., 1945, 24, 648.
12. Rutstein, D. D., Clarke, F. H., and Taran, L. M., Electrophoretic studies in rheumatic fever. Science, 1945, 101, 670.
13. van der Scheer, J., Lagsdin, J. B., and Wyckoff, R. W. G., Electrophoretic and ultracentrifugal analyses of antipneumococcal horse sera. J. Immunol., 1941, 41, 209.
14. Blix, G., Tiselius, A., and Svensson, H., Lipids and polysaccharides in electrophoretically separated blood serum proteins. J. Biol. Chem., 1941, 137, 485.
15. Nilsson, Ivar, Über den glucosamingehalt des blutserums bei gesunden und pneumoniekranken. Biochem. Z., 1937, 291, 254.
16. Lustig, B., and Langer, A., Über die Bestimmung und den Gehalt an freiem, Eiweiss- und gebundenem Nichteiweisszucker in normalen und pathologischen Serum, Liquor und Harn. Biochem. Z., 1931, 242, 320.
Lustig, B., and Nassau, E., The carbohydrate content of the proteins in blood serum, cantharidin blisters and pleural effusions. Am. Rev. Tuberc., 1941, 43, 817.
17. Friedmann, T. E., and Sutliff, W. D., Appearance of fermentable polysaccharide in the blood and a simple method of its detection. Science, 1939, 90, 335.
18. Seibert, F. B., Nelson, J. W., and Seibert, M. V., Correlation of extent of tuberculosis with amount of polysaccharide in the serum. Proc. Soc. Exper. Biol. and Med., 1943, 52, 219.
19. National Tuberculosis Association, Diagnostic Standards and Classification of Tuberculosis, 1940 Edition, New York City.
20. Seibert, F. B., and Atno, J., Determination of polysaccharide in serum. J. Biol. Chem., 1946, 163, 511.
21. Cutler, J. W., Standardization of sedimentation rate. Am. Rev. Tuberc., 1943, 48, 314.
22. Somogyi, M., A reagent for the copper-iodometric determination of very small amounts of sugar. J. Biol. Chem., 1937, 117, 771.
23. Kingsley, G. R., The direct biuret method for the determination of serum proteins as applied to photoelectric and visual colorimetry. J. Lab. and Clin. Med., 1942, 27, 840.
24. Seibert, F. B., and Nelson, J. W., Electrophoretic study of the blood protein response in tuberculosis. J. Biol. Chem., 1942, 143, 29.
25. Longsworth, L. G., Recent advances in the study of proteins by electrophoresis. Chem. Rev., 1942, 30, 323.
26. Perlmann, G. E., and Kaufman, D., The effect of ionic strength and protein concentration in the electrophoretic analysis of human plasma. J. Am. Chem. Soc., 1945, 67, 638.
27. Svensson, Harry, Electrophoresis by the Moving Boundary Method. A theoretical and experimental study. Arkiv. for Kemi, Mineralogi och Geologi, 1946, 22A, No. 10 (Thesis).
28. Svensson, H., Direkte photographische Aufnahme von Elektrophorese-Diagrammen. Kolloid.-Z., 1939, 87, 181. Theorie der Beobachtungsmethode der gekreuzten Spalte. Kolloid.-Z., 1940, 90, 141.
29. Longsworth, L. G., and MacInnes, D. A., Electrophoresis of proteins by the Tiselius method. Chem. Rev., 1939, 24, 271.
30. Svedberg, T., and Pedersen, K. O., The Ultracentrifuge, p. 296. Oxford University Press, London, 1940.
31. Dole, Vincent P., The electrophoretic patterns of normal plasma. J. Clin. Invest., 1945, 23, 708.
32. Longsworth, L. G., Curtis, R. M., and Pembroke, R. H., Jr., The electrophoretic analysis of material and fetal plasmas and sera. J. Clin. Invest., 1945, 24, 46.
33. Gavett, G. I., A First Course in Statistical Method, p. 188. McGraw-Hill Book Co., Inc., New York, 1937.
34. Enders, J. F., The concentration of certain antibodies in globulin fractions derived from human blood plasma. J. Clin. Invest., 1944, 23, 510.
35. Seibert, F. B., and Nelson, J. W., Proteins of tuberculin. J. Am. Chem. Soc., 1943, 65, 272.
36. Emmart, E. W., and Seibert, F. B., The effect of tuberculous and sensitized sera and serum fractions on the development of tubercles in the chorio-

allantoic membrane of the chick. J. Immunol., 1945, 50, 143.

37. Shedlovsky, T., and Scudder, J., A comparison of erythrocyte sedimentation rates and electrophoretic patterns of normal and pathological human blood. J. Exper. Med., 1942, 75, 119.

38. Perlmann, G. E., Glenn, W. W. L., and Kaufman, D., Changes in the electrophoretic pattern in lymph and serum in experimental burns. J. Clin. Invest., 1943, *22*, *627*.

39. Zeldis, L. J., and Alling, E. L., Plasma protein metabolism-electrophoretic studies. Restoration of circulating proteins following acute depletion by plasmapheresis. J. Exper. Med., 1945, 81, 515.

THE ANEMIA OF INFECTION. III. THE UPTAKE OF RADIOACTIVE IRON IN IRON-DEFICIENT AND IN PYRIDOXINE-DEFICIENT PIGS BEFORE AND AFTER ACUTE INFLAMMATION [1]

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In the first (1) of this series of studies on the pathogenesis of the anemia of infection, it was pointed out that there must be in association with infection a derangement in the intermediate metabolism of iron. This conclusion was based on the observation that in cases of infection associated with anemia, the plasma iron content is markedly lowered below the normal; this appears early, and is found even before anemia has developed. The injection of staphylococci or of turpentine in dogs was shown (2) to be associated with hypoferremia, and evidence was presented (1) which indicated that absorption of iron is probably adequate when infection is present, the hypoferremia being accompanied by a rapid removal of iron from the plasma. The present report, and those which follow, describe experiments which were designed to study the nature of the disturbance in the internal metabolism of iron.

When iron deficiency is present, anemia develops which is accompanied by marked hypoferremia. If iron is then given intravenously (3) it is taken up rapidly and quantitatively for erythropoiesis. The anemia associated with pyridoxine deficiency, on the other hand, is characterized by inability to use iron, with the result that it is deposited in the tissues and marked hyperferremia develops (4). Thus these conditions represent wide extremes in the ability to use iron for hemoglobin formation. It was thought that some insight might be gained as to the nature and severity of the alteration in iron metabolism associated with infection if hemoglobin synthesis and the uptake of radioactive iron by the red corpuscles were studied before and after acute inflammation was induced in these widely different types of anemia.

To measure the capacity to utilize iron for hemoglobin synthesis, 2 widely different quantities of radioactive iron (Fe^{59}) have been injected intravenously in the experimental animals. The smaller amount, 10 to 17 μg. per kgm. body weight, corresponds to the quantity which, in the normal human, may possibly be absorbed each day (5), namely 0.7 to 1.2 mgm. This is considerably lower than the amount of iron liberated from hemoglobin breakdown per day, which may amount to 25 mgm. The larger dose of Fe^{59} we have employed, 225 to 330 μg. per kgm. body weight, corresponds approximately to the amount derived each day from hemoglobin catabolism. Provided iron metabolism in the pig is similar to that in man, the smaller injection of Fe^{59} may therefore be regarded as much below the amount which the hemopoietic system can use, while the larger amount slightly taxes the metabolic system, since it presumably doubles, approximately, the quantity of iron which is available for the formation of hemoglobin.

In order to make certain that the radioactive iron appearing in the blood was present in hemoglobin, in a number of instances hemin was crystallized from the blood of animals which had received Fe^{59}. The radioactivity of the crystalline material was then compared with the activity in the whole blood per mgm. of iron.

METHODS

The experimental animals were growing pigs, similar to those used in studies already reported from this laboratory (6).

Inflammation or infection was produced in the following manner. Sterile abscesses were produced by intramuscular or subcutaneous injections of 4 ml. of turpen-

[1] The work described in this paper was commenced under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Utah. The expenses of this investigation were also defrayed in part by grants from the United States Public Health Service, Parke, Davis and Company and the Upjohn Company.

tine. Bacterial infection was induced by the daily intramuscular injection of 5 ml. of a mixed culture of *S. aureus* and *E. coli*. To produce burns, the bottom of a 125 ml. Erlenmeyer flask containing boiling water was placed on the flank of the animal for 20 seconds.

For intravenous injection the radioactive iron was prepared in the following manner. An aliquot of the Fe^{59}, obtained as $FeCl_3$ in hydrochloric acid, was reduced with ascorbic acid. To this 2 N sodium hydroxide was added drop by drop, until the solution just became violet brown. The solution was then diluted to volume with water. If a greater quantity of iron per unit volume was desired, non-radioactive $FeCl_3$ was added before reduction with ascorbic acid. The purity of the radioactive iron employed was ascertained by the rate of decay.

Hemin was isolated from blood samples by the method of Schalfejeff (8) as follows. A sample of blood cells was allowed to drip for a period of 15 minutes from a small separatory funnel into a 50 ml. centrifuge tube containing 3 volumes of glacial acetic acid saturated with sodium chloride. The tube was kept at 90 to 95° C. by using a hot plate, and the solution was agitated constantly with an electric stirrer. After it had cooled to room temperature, the material was allowed to stand over night in the refrigerator. The hemin was centrifuged down and, in sequence, it was washed with 50 per cent acetic acid, distilled water, 95 per cent ethyl alcohol (twice) and ether (twice). The yield of crystalline hemin was 50 to 70 per cent. In order to determine the quantity of hemin, the dry material was dissolved in a known volume of 0.1 NaOH, and a dilution of this solution was read in the photoelectric colorimeter at 520 mμ using a standard curve constructed with hemin of known purity as determined by iron measurement and spectrophotometric analysis. The hemin solutions were then digested and prepared for counting just as in the case of blood. Along with the hemin isolation and analysis, Fe^{59} analyses were made of the same original blood, the plasma being removed prior to preparation.

Hemoglobin determinations were carried out on the samples taken for Fe^{59} estimation. The volume of blood used was noted in order to calculate the number of grams of hemoglobin analyzed. The photoelectric oxyhemoglobin method of Bell, Chambers and Waddell (9) was employed, the instrument having been standardized by several procedures, including the oxygen capacity method (10) and the hemin method (11).

Calculation of Fe^{59} uptake. In studies with human subjects the uptake of Fe^{59} is usually calculated as follows (12):

$$\text{Total circulating hb.} = \frac{\text{blood volume}}{100} \times \text{grams hb. per 100 ml. blood;}$$

$$\text{Circulating counts} = \text{total circulating hb} \times \text{counts per gram hb.}$$

Many workers have assumed that the total blood volume is 80 ml. per kgm. body weight, believing that this assumption is at least as accurate as most total blood volume estimations made by calculation from the measured plasma volume and volume of packed cells (12).

In the case of growing pigs we have encountered considerable difficulty because of obvious shifts in plasma volume. In such pigs, especially in anemic animals, the hemoglobin per 100 ml. of blood shows wide and rapid variations. As a result, with sharp changes of hemoglobin one may get such impossible values as negative uptakes well beyond the range of error. In order to overcome this difficulty, we have devised a method of calculation which is based on the following assumptions.

(*1*) The total circulating hemoglobin is relatively constant, or increases or decreases consistently, and under our experimental conditions does not vary as much as is indicated by the changes in hemoglobin per 100 ml. of blood.

(*2*) The variations of the total circulating hemoglobin as obtained by the formula,

$$\frac{\text{weight of animal (kgm.)} \times 80}{100} \times \text{hb. per 100 ml.,}$$

range about the true value in a homeostatic manner. Accordingly, in these studies the body weight and the hemoglobin, in grams per 100 ml. of blood, have been plotted for each pig against time. From these values the total circulating hemoglobin has been calculated, and this also has been plotted on the graph. A line has then been drawn through the estimated average of the plotted values for the total circulating hemoglobin. It is assumed that this line presents a picture of the true alterations in total circulating hemoglobin. When for the calculation of Fe^{59} uptake it has been necessary to know the total circulating hemoglobin, the value has been taken from the appropriate point on this mean curve.

In the case of iron-deficient pigs, the actual mass of circulating hemoglobin was also determined by assuming that in such animals Fe^{59} is completely used for hemoglobin formation in 7 to 14 days or less. The following formula may be applied when Fe^{59} utilization is complete:

$$\frac{\text{Total counts injected intravenously}}{\text{counts per gram hemoglobin}} = \text{total circulating hb.}$$

The correlation of the estimated values for total circulating hemoglobin with those obtained by the radioactive method is shown in Figure 1. Since both methods entail sources of error the degree of correlation found is of interest.

RESULTS

Figure 2 illustrates the type of uptake curves obtained in different pigs. The complete data are recorded in Tables I to IV.

It will be observed that the iron-deficient animals usually showed rapid uptake, with little difference in the degree of uptake of the small and large doses of iron. This was to have been expected, since it is well known that such animals

can utilize iron very rapidly, even when it is given in large quantities. On the other hand the pyridoxine-deficient pigs were able to use a good percentage of the small dose only, a negligible percentage of the larger dose being used. In terms of absolute quantities of iron, the uptake was of the same order of magnitude when the large or the small dose was given. In the pyridoxine-deficient pig, hemoglobin formation is limited because of deficiency of this vitamin (4). That the iron not used in hemoglobin formation is stored, is indicated by the observations illustrated in Figure 3,

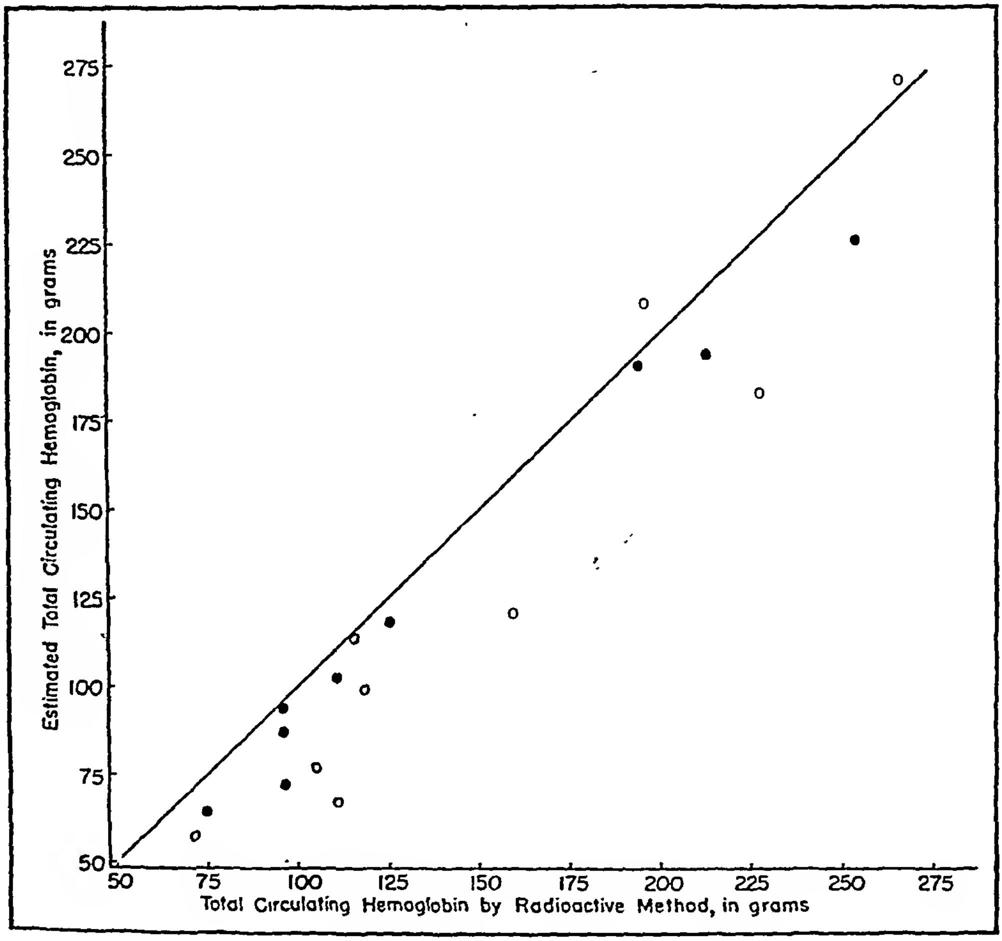

Fig. 1. The Correlation of Total Circulating Hemoglobin as Estimated from a Line Best Fitting the Plotted Values for Total Circulating Hemoglobin (see text) ("Estimated Total Circulating Hemoglobin") with the Total Circulating Hemoglobin as Determined From the Dilution of Radioactive Iron Injected Intravenously in Iron-Deficient Pigs

The closed circles represent the correlation with the values based on the first injection of radioactive iron, and the open circles refer to values after a second injection. In these calculations the total amount of radioactive iron injected in the animal has been divided by the number of counts per gram. The necessity for assuming that all of the injected Fe^{59} has been incorporated in the hemoglobin, even when more than one injection has been made, limits the accuracy of this method. The trend for higher values for total circulating hemoglobin, as measured by the radioactive method as compared with those based on the estimated values, can be explained, at least in part, by failure to use Fe^{59} completely for hemoglobin formation. Consistent with this view is the fact that the best correlation was found with the values obtained after the first injection of radioactive iron, when the likelihood of complete utilization of iron for hemoglobin formation was greater.

in which Fe^{59} uptake by the red cells is seen to increase sharply when pyridoxine is supplied.

The uptake of iron by the red cells of the normal animals was intermediate between that observed in the pyridoxine-deficient and the iron-deficient pigs. In the normal mature pig the small dose was used more rapidly than the large one.

Figure 4 shows the effect of acute inflammation on the rate of hemoglobin formation in iron-deficient pigs. It will be seen that 5 of the 6 pigs showed retarded hemopoiesis. Since the otherwise normal iron-deficient animal is capable of completely utilizing for hemoglobin synthesis much larger doses of Fe^{59} than those used in this experiment, retardation of the uptake of 10 μg. Fe^{59} per kgm. must be regarded as indicating severe depression of hemopoietic function. Pig 9–24, in Figure 2, is an example of the effect of a more chronic infection on the rate of uptake of Fe^{59}. This animal had been attacked by a dog and received severe lacerations on the legs which subsequently became chronically infected. It may be noted that in this pig a larger dose of Fe^{59} was utilized even more poorly than the smaller one.

Figure 5 illustrates the extent to which infection impairs the response to therapy with iron in iron-deficient pigs. Two iron-deficient pigs, one of which was receiving daily injections of a bacterial

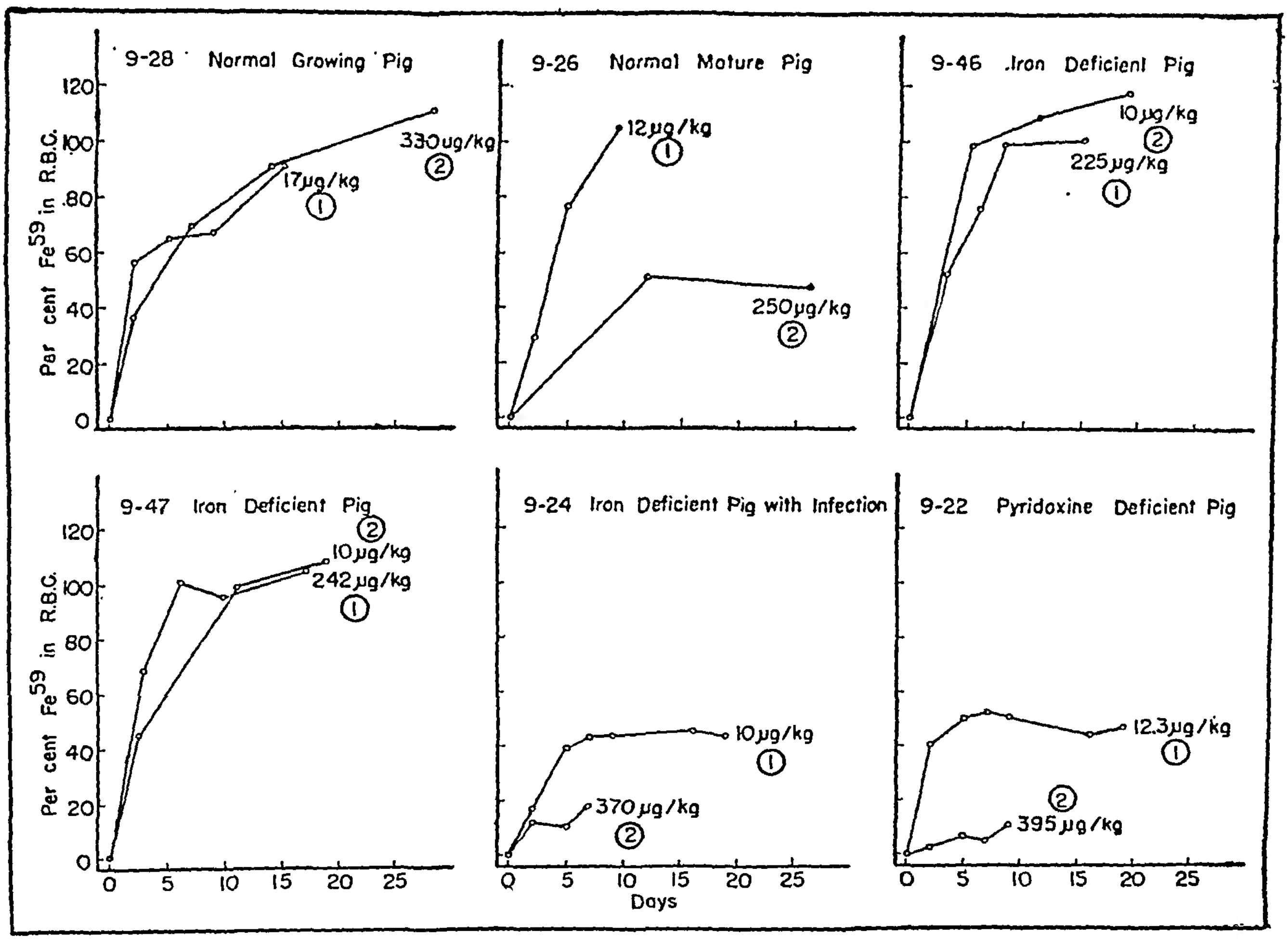

FIG. 2. UPTAKE OF INTRAVENOUSLY INJECTED RADIOACTIVE IRON (Fe^{59}) BY RED CELLS OF NORMAL GROWING AND MATURE PIGS AND IN PIGS WITH IRON DEFICIENCY OR PYRIDOXINE DEFICIENCY

A small and a large amount of iron was given in each instance. The order in which these were given is indicated as (*1*) and (*2*).

Note the more rapid uptakes of iron by the iron-deficient animals (9–46, 9–47) as compared with the normal ones, the less complete uptake of the large dose by the normal mature pig (9–26) as compared with the growing normal pig (9–28), and the poor uptake of iron by the pyridoxine-deficient pig (9–22).

The marked impairment of uptake of iron produced by infection in an iron-deficient pig (9–24) as compared with the uptake in a similar animal without infection (9–47), should be noted.

TABLE I

Uptake of small doses of intravenously injected Fe^{59} by the red corpuscles of normal and of pyridoxine-deficient pigs

Pig number	Normal					Pyridoxine deficient			
	9–25	9–26	9–28	9–29	9–30	9–22	9–39	9–40	9–40
Age, days	300	263	146	146	146	146	66	66	80
Weight (kgm.)	97.0	75.0	43.1	38.0	37.1	22.0	14.3	11.6	14.7
Previous injections of Fe^{59}, number	3	2	0	0	0	0	0	0	1
Previous injections of Fe^{59}, amount (mgm.)	40.83	12.57	0	0	0	0	0	0	0.12
Days since previous injection	37	96	0	0	0	0	0	0	14
Fe^{59} injected, amount (mgm.)	0.97	0.90	0.74	0.60	0.62	0.27	0.14	0.12	0.15
Days after injection	**Percentage uptake**								
2	34.1	28.3	56.7	26.3		41.5	5.0	43.0	
3									21.3
5	75.4	76.7	64.7	42.6	60.6	50.1	15.3	52.5	
7				45.9	62.8	52.6			
9		105.0	66.9	40.5	67.0	50.6	13.3	63.0	
11								45.8	
12	51.3								
15			92.0	55.1					
16					69.4	44.5			
19				65.3	67.1	47.1			
25									31.2
26	65.5								
28									42.0

TABLE II

Uptake of large doses of intravenously injected Fe^{59} by the red corpuscles of normal and of pyridoxine-deficient pigs

Pig number	Normal					Pyridoxine deficient		
	9–25	9–26	9–28	9–29	9–30	9–22	9–42	9–42
Age, days	263	300	167	167	169	166	66	80
Weight (kgm.)	83.6	99.4	56.2	49.7	48.3	26.6	9.0	10.9
Previous injections of Fe^{59}, number	2	3	1	1	1	1	0	1
Previous injections of amount (mgm.)	19.83	13.47	0.74	0.60	0.62	0.27	0	2.25
Days since previous injection	96	96	21	21	21	21	0	14
Fe^{59} injected, amount (mgm.)	21.0	24.8	18.55	8.65	15.95	7.80	2.25	2.73
Days after injection	**Percentage uptake**							
2	0		36.4	25.5	34.4	2.2	1.6	0
4								10.3
5	72.8				29.5	6.9	8.0	
7			69.5	61.0	59.0	5.0		
9	58.2					10.2	5.5	
11								10.8
12		50.8						
14			91.0	98.5	81.7		5.3	
25								6.8
26		46.7						
28			111.0	127.6				19.4
36					92.8			

TABLE III

Uptake of small doses of intravenously injected Fe^{59} by the red corpuscles of iron-deficient pigs

Pig number	9-25	9-26	9-27	9-46	9-47	9-48	9-49	9-50	9-51
Age, days	146	146	146	84	84	84	67	67	67
Weight (kgm.)	36.8	29.3	37.9	25.9	27.0	25.2	20.0	23.7	34.5
Previous injections of Fe^{59}, number	0	0	0	1	1	1	0	0	0
Previous injections of Fe^{59}, amount (mgm.)	0	0	0	5.75	6.75	5.23	0	0	0
Days since previous injection	0	0	0	17	17	17	0	0	0
Fe^{59} injected, amount (mgm.)	0.48	0.27	0.40	0.26	0.27	0.25	0.20	0.24	0.35
Days after injection	**Percentage uptake**								
2	22.2	37.0	33.0	37.8	46.7	84.0			
3							82.6	59.0	66.0
5	52.2	54.1	42.2	98.4		127.6			
6							74.7	76.8	84.0
7	63.0	65.4	42.2						
9	57.0	92.5	58.7						
10							89.0		
11				110.0	98.5	134.0			
15	92.0		63.2						
17								97.0	98.5
19	88.7		90.5	120.0	107.0	138.0			

TABLE IV

Uptake of large doses of intravenously injected Fe^{59} by the red corpuscles of iron-deficient pigs

Pig number	9-25	9-26	9-27	9-46	9-47	9-48	9-49	9-50	9-51
Age, days	167	167	167	67	67	67	84	84	84
Weight (kgm.)	46.4	37.2	48.3	23.0	23.3	20.9	21.3	28.2	42.7
Previous injections of Fe^{59}, number	1	1	1	0	0	0	1	1	1
Previous injections of Fe^{59}, amount (mgm.)	0.48	0.27	0.40	0	0	0	0.20	0.24	0.35
Days since previous injections	21	21	21	0	0	0	17	17	17
Fe^{59} injected, amount (mgm.)	19.35	12.30	16.17	5.24	5.65	4.89	5.33	7.05	10.70
Days after injection	**Percentage uptake**								
2	59.0	28.8	33.9				48.3	43.8	60.0
3				53.3	69.5	82.3			
4	75.7								
5		55.8	65.3				65.2	58.5	102.0
6	91.2			77.0	100.5	102.0			
7			86.2						
8	99.0			100.0					
9		61.5	83.5						
10					95.5	109.0			
11							79.0	75.6	120.0
13	112.0								
14		62.5							
15				102.0					
17					104.0				
19								84.3	101.0

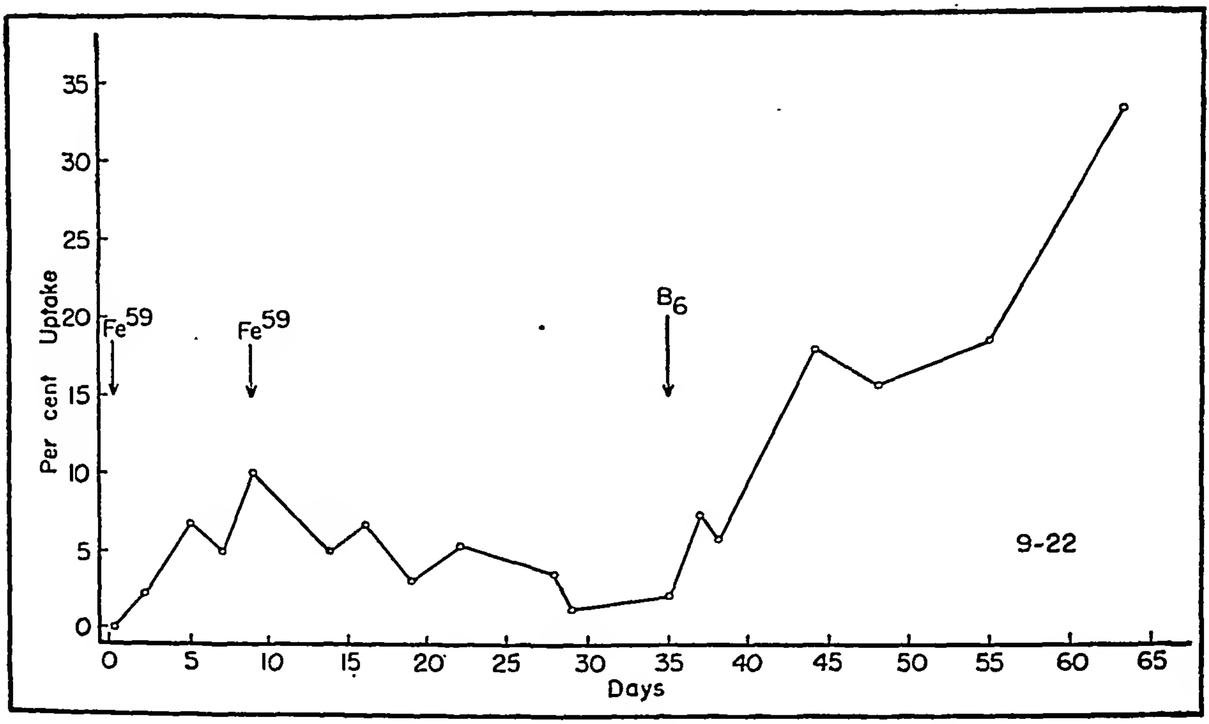

FIG. 3. UPTAKE OF INTRAVENOUSLY INJECTED RADIOACTIVE IRON (Fe^{59}) BY THE RED CELLS OF A PYRIDOXINE-DEFICIENT PIG BEFORE AND AFTER THE ADMINISTRATION OF PYRIDOXINE (B_6)

Two injections of Fe^{59}, each 250 μg. per kgm. body weight, were made. The percentage uptake has been calculated on the basis of the sum of the 2 doses.

That the iron which failed to enter the red cells before pyridoxine was given had been stored, is indicated by the appearance of the radioactive iron in the red cells after normal hemoglobin formation was made possible by the administration of pyridoxine.

mixture (*S. aureus* and *E. coli*), were both given intravenously 100 mgm. of iron per day for 4 days. The uninfected pig (9–49) showed prompt and rapid hemoglobin synthesis. From calculations of the total circulating hemoglobin before and after treatment, it is estimated that 83 per cent of the iron was utilized for hemoglobin formation. On the other hand, the animal receiving the bacterial mixture did not respond significantly to iron therapy until the bacterial injections were stopped and penicillin was used.

In Figure 6 is shown the effect of infection on the response of pyridoxine-deficient pigs to vitamin B_6. One pyridoxine-deficient pig (9–40) was given daily intramuscular injections of a bacterial mixture. Both were given pyridoxine intramuscularly. As will be seen, the animal receiving the bacterial mixture formed hemoglobin but slowly, even though large amounts of pyridoxine were given, whereas the non-infected pig synthesized hemoglobin promptly.

In Table V is presented evidence that Fe^{59} appearing in the blood cells is present as hemoglobin iron. The considerable variation in some instances, well beyond the usual error in the recovery and counting of Fe^{59} in a sample of blood (7), may be ascribed to the small quantity of hemin isolated and thus to the unusually small number of counts with which we were dealing. In spite of this technical difficulty, it will be noted that the average values for these animals showed approximately 100 per cent of the red cell Fe^{59} to be hemoglobin iron. The procedure employed for isolating and purifying the hemin could not possibly have carried through Fe^{59} which was not in the hemoglobin structure originally.

DISCUSSION

It is clear from the present studies that marked retardation of hemopoiesis occurs as a result of inflammation. This is in agreement with the findings of Robscheit-Robbins and Whipple (13)

who showed that a sterile abscess will diminish the production of new hemoglobin in the anemic dog when liver is being fed. That so powerful a stimulus as intravenous iron therapy for an iron- deficient animal, or pyridoxine for a B_6-deficient pig, cannot overcome the retardation or does so slowly and incompletely, attests to the severity of the effect of inflammation. Hemoglobin production normally has priority over other protein production (14). Any process which has precedence over hemoglobin formation can be expected to be important to the survival of the animal.

There is no reason to assume that the mechanism whereby hemoglobin formation is retarded by acute inflammation is different from that associated with chronic infection, even though anemia more commonly accompanies the latter than the former. That time is required for anemia to develop suggests that blood destruction is not a significant factor; instead, as red corpuscles wear out, they fail to be replaced. The "life cycle" of the red corpuscle is thought to be about 125 days (15).

It is of interest in connection with the pathogenesis of the anemia of infection that the pyridoxine-deficient pig which represents, in a sense, a state of supersaturation with iron, is incapable of synthesizing hemoglobin efficiently in the presence

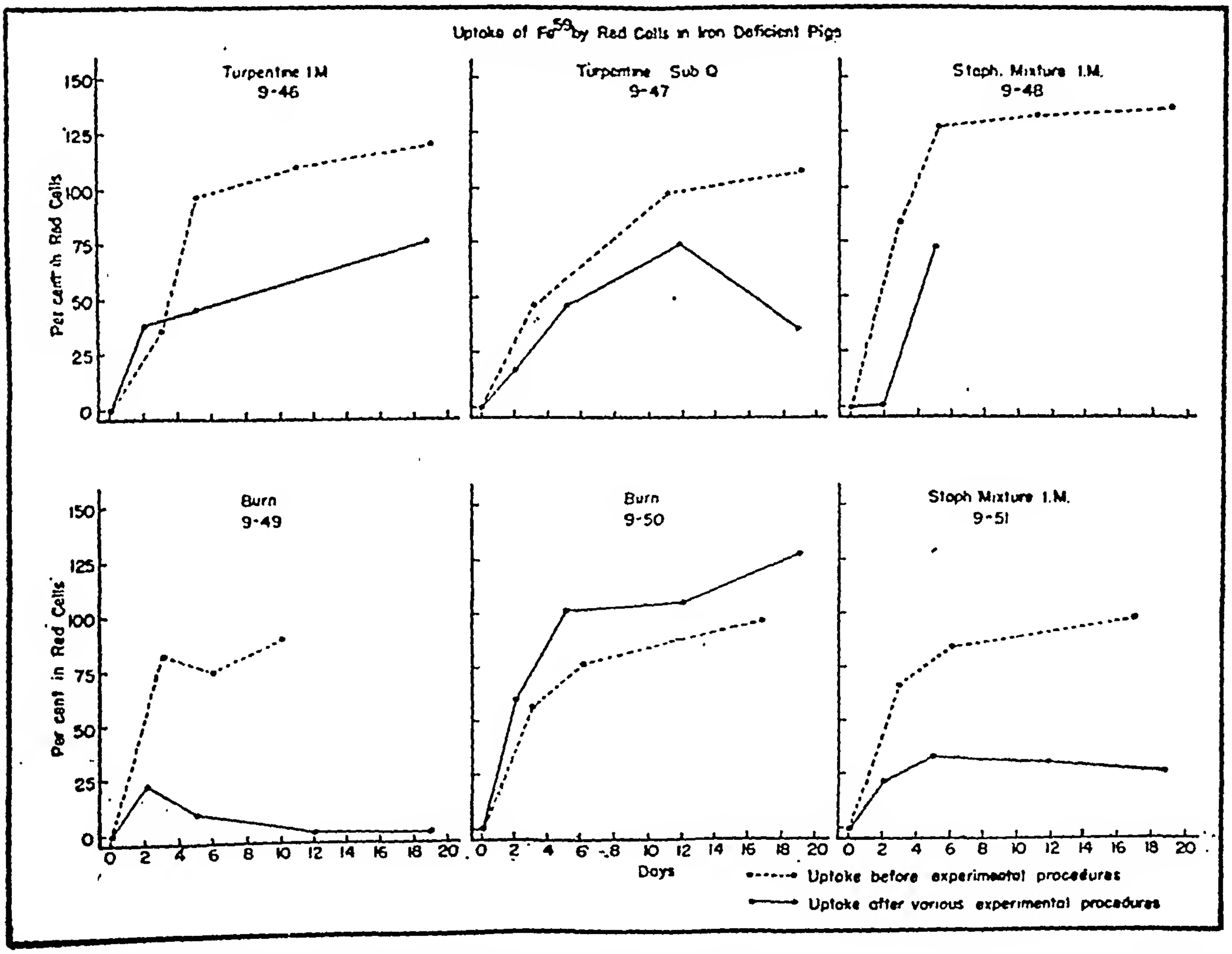

FIG. 4. THE INFLUENCE OF ACUTE INFLAMMATION ON THE UPTAKE OF INTRAVENOUSLY INJECTED RADIOACTIVE IRON BY THE RED CELLS OF IRON-DEFICIENT PIGS

In all instances small amounts of iron were given (10 μg. per kgm. body weight). The interrupted lines represent the uptake before the experimental procedure was carried out and the continuous line indicates the uptake after inflammation was produced.

Note that in every instance but one, a marked retardation of uptake occurred whether turpentine or a bacterial culture was injected, or a burn was produced.

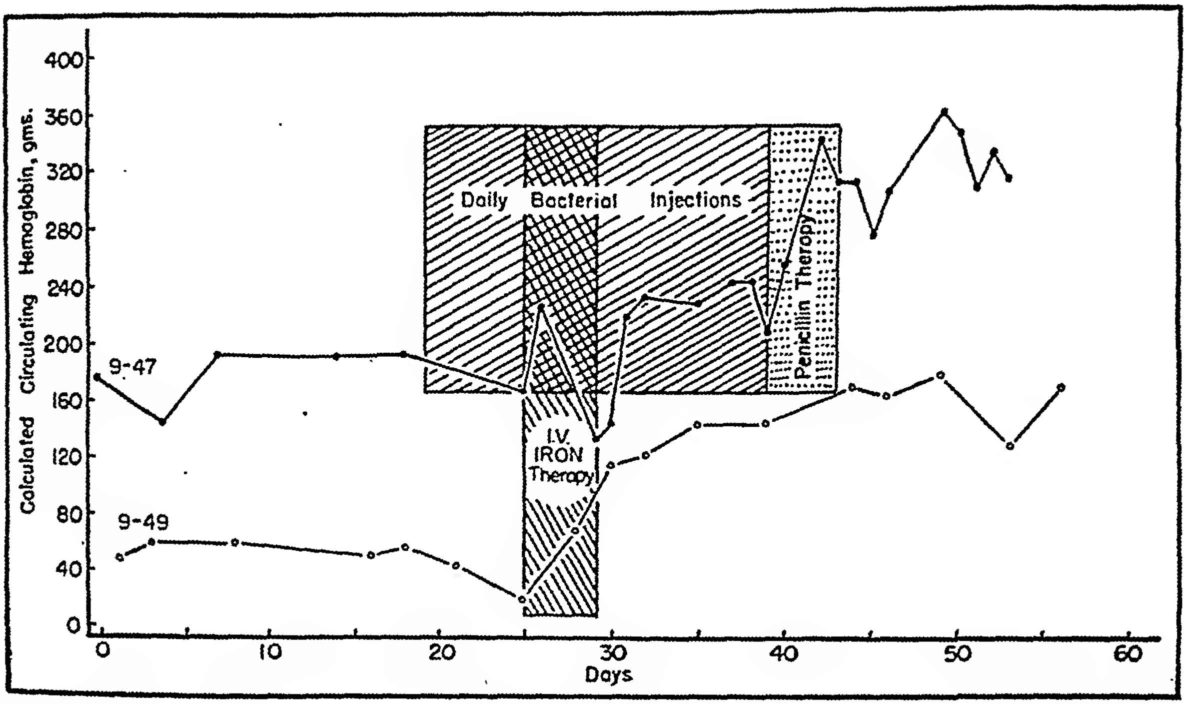

FIG. 5. THE EFFECT OF INFLAMMATION PRODUCED BY DAILY BACTERIAL INJECTIONS ON THE HEMOPOIETIC RESPONSE OF IRON-DEFICIENT PIGS TO THE ADMINISTRATION OF IRON

In the non-infected animal (9–49) the intravenous administration of 400 mgm. iron, given as ferric chloride reduced by ascorbic acid, and then brought to neutrality with alkali, was followed by a prompt and almost complete utilization of the iron for hemoglobin formation as estimated from the rise in calculated total circulating hemoglobin. In the infected animal, on the other hand, the rise above the average level preceding iron administration was comparatively small until the injections were discontinued, the abscesses were drained, and penicillin was given. Following such treatment the hemoglobin rose to the expected level.

of infection, even when pyridoxine is given. This suggests that a limitation of iron is not the primary cause of the anemia of infection. In the paper (16) which follows, additional evidence is presented which indicates that this anemia does not result from a lack of iron.

Comment may be made concerning the usefulness of the radioactive technic as a measure of retarded hematopoiesis. Observation of the rate and extent of the uptake of intravenously administered Fe^{59} is useful, particularly, in circumstances in which retarded hemoglobin formation is suspected but anemia is not present. Theoretically, at least, it should also be helpful in the study of cases where diminished red cell destruction exists to compensate for impaired hemoglobin formation. In using radioactive iron for such purposes, it is important to employ such amounts as will test the functional efficiency of the hemopoietic system, and yet will not be so great that the major portion of the iron given must necessarily be shunted into the iron stores.

SUMMARY

1. The uptake of radioactive iron, injected intravenously, has been studied in normal growing and mature pigs, in iron-deficient and in pyridoxine-deficient pigs, and in such animals when inflammation was produced by various means.

2. For this purpose, 2 different quantities of iron were used, one corresponding to the amount assumed to be absorbed daily from the diet, the other corresponding to the estimated amount derived from normal hemoglobin catabolism. These quantities have been selected in order to test the functional capacity of the hemopoietic system.

3. It has been shown by the isolation of hemin that the radioactive iron appearing in the blood is present in the hemoglobin moiety.

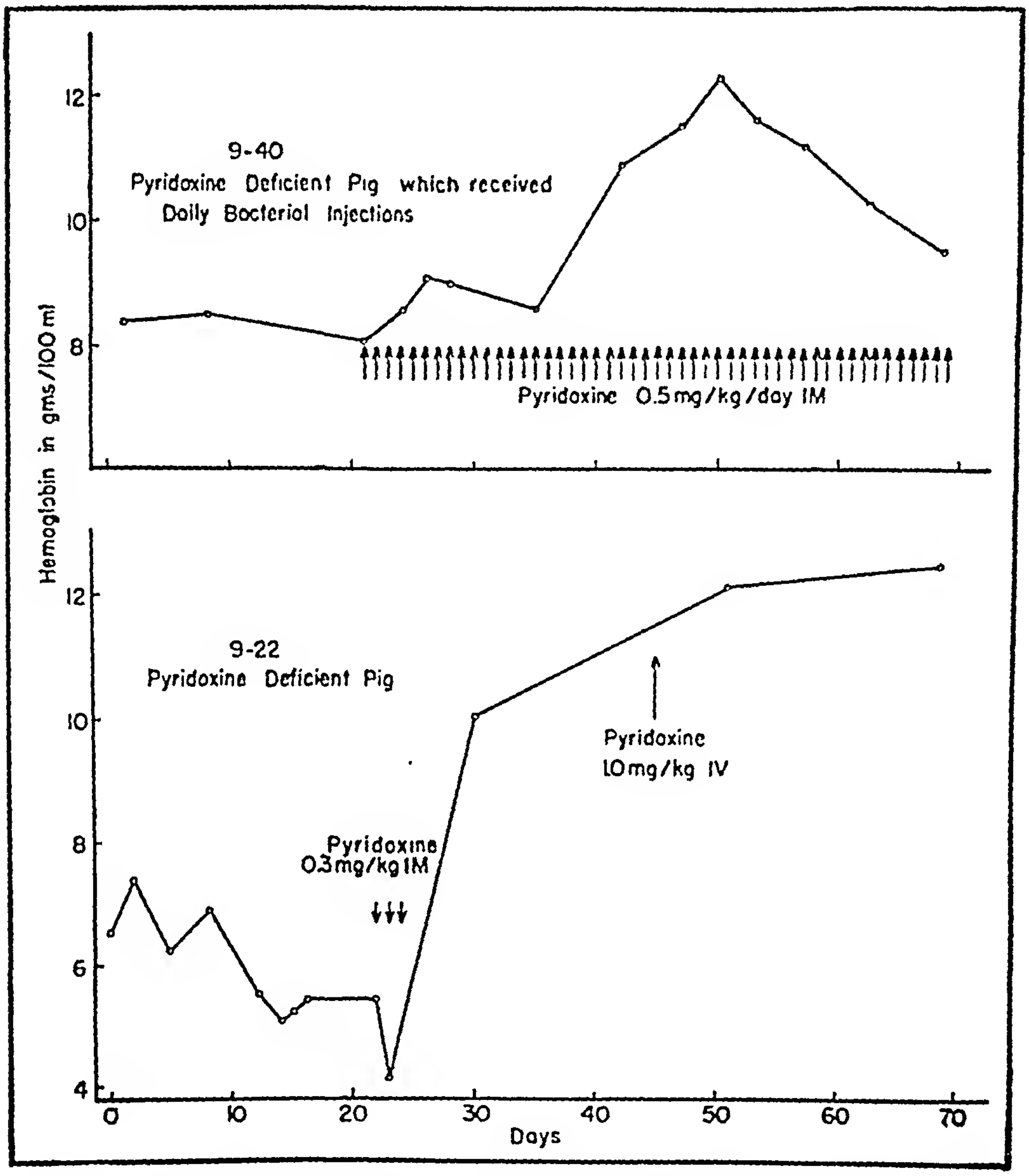

FIG. 6. THE EFFECT OF INFLAMMATION PRODUCED BY DAILY INTRAMUSCULAR INJECTIONS OF BACTERIAL CULTURES ON THE HEMOPOIETIC RESPONSE OF PYRIDOXINE-DEFICIENT PIGS TO THE ADMINISTRATION OF PYRIDOXINE

In the non-infected animal (9–22) the injection of pyridoxine was followed by a prompt and marked rise in hemoglobin, whereas in the pig in which infection was produced (9–40), the response was slow, small in amount, and was not maintained, even though much larger amounts of pyridoxine were given.

4. It has been demonstrated that the uptake of radioactive iron is rapid and complete in iron deficiency; slow, and even negligible, if large doses are used, in pyridoxine deficiency; and intermediate, between these extremes in normal animals.

5. In spite of the pronounced avidity of the iron-deficient animal for iron, in the presence of inflammation the uptake of iron is markedly impaired.

6. In pyridoxine deficiency the rapid and efficient uptake of iron which follows the administration of pyridoxine, is markedly reduced by the presence of infection.

7. It is concluded that the anemia of infection is caused by impaired hemoglobin production. Anemia does not appear at once, however, for it is only when outworn red corpuscles must be replaced that the defect is noticeable.

The radioactive iron used in these studies was obtained from the Massachusetts Institute of Technology through the courtesy of Dr. Robley D. Evans.

TABLE V

Conversion of intravenously injected Fe^{59} to hemoglobin iron in pigs

Animal no.	Status	Day following Fe^{59} injection	Percentage uptake of Fe^{59} by RBC	Percentage of RBC Fe^{59} which is hemoglobin iron
9-22	Pyridoxine deficient	5 7 9	50 53 51	101 101 116
9-24	Iron deficient + infection	7 9	43 43	106 119
9-26	Iron deficient	2 5 7	37 54 65	104 122 97
9-30	Normal	5	61	126
9-42	Pyridoxine deficient	2	24	102
9-46	Iron deficient	2	62	89
9-47	Iron deficient	2	37	109
9-49	Iron deficient	2	59	103
9-50	Iron deficient	2	62	95
Average Fe^{59} as hemoglobin iron				107

Calculations;

a. Counts per mgm. hb. iron

$$= \frac{\text{Counts per gram hb. in original blood}}{3.39}.$$

b. Counts per mgm. hemin iron

$$= \frac{\text{Counts per 10 mgm. crystallized hemin}}{0.86}.$$

c. Percentage of RBC Fe^{59} which is hemoglobin iron

$$= \frac{b}{a} \times 100.$$

BIBLIOGRAPHY

1. Cartwright, G. E., Lauritsen, M. A., Jones, P. J., Merrill, I. M., and Wintrobe, M. M., The anemia of infection. I. Hypoferremia, hypercupremia and alterations in porphyrin metabolism in patients. J. Clin. Invest., 1946, 25, 65.
2. Cartwright, G. E., Lauritsen, M. A., Humphreys, S. R., Jones, P. J., Merrill, I. M., and Wintrobe, M. M., The anemia of infection. II. The experimental production of hypoferremia and anemia in dogs. J. Clin. Invest., 1946, 25, 81.
3. Wintrobe, M. M., Greenberg, G. R., Humphreys, S. R., and Kramer, R., The development of anemia in growing pigs receiving an iron-deficient diet (to be published).
4. Wintrobe, M. M., Follis, R. H., Jr., Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S. R., Suksta, A., and Cartwright, G. E., Pyridoxine deficiency in swine. Bull. Johns Hopkins Hosp., 1943, 72, 1.
5. Moore, C. V., Dietotherapy or Clinical Application of Modern Nutrition. M. Wohl, Saunders Co., Philadelphia, 1945.
6. Wintrobe, M. M., Miller, J. L., Jr., and Lisco, H., The relation of diet to the occurrence of ataxia and degeneration in the nervous system of pigs. Bull. Johns Hopkins Hosp., 1940, 67, 377.
7. Greenberg, G. R., Humphreys, S. R., Ashenbrucker, H., Lauritsen, M., and Wintrobe, M. M., A simple apparatus and procedure for preparing and electroplating radioactive iron. Blood, 1947, 2, 94.
8. Gatterman, L., and Wieland, H., Laboratory Methods of Organic Chemistry. Eng. Trans., 1937, Macmillan Co., New York, p. 407.
9. Bell, G. H., Chambers, J. W., and Waddell, M. B. R., The routine estimation of haemoglobin as oxyhaemoglobin. Biochem. J., 1945, 39, 60.
10. Van Slyke, D. D., and Stadie, W. C., The determination of the gases of the blood. J. Biol. Chem., 1921, 49, 1.
11. Clegg, J. W., and King, E. J., Estimation of hemoglobin by alkaline haematin method. Brit. Med. J., 1942, 2, 329.
12. Moore, C. V., Dubach, R., Minnich, V., and Roberts, H. K., Absorption of ferrous and ferric radioactive iron by human subjects and by dogs. J. Clin. Invest., 1944, 23, 755.
13. Robscheit-Robbins, F. S., and Whipple, G. H., Infection and intoxication. Their influence upon hemoglobin production in experimental anemia. J. Exper. Med., 1936, 63, 767.
14. Robscheit-Robbins, F. S., Madden, S. C., Rowe, A. P., Turner, A. P., and Whipple, G. H., Hemoglobin and plasma protein. Simultaneous production during continued bleeding as influenced by diet protein and other factors. J. Exper. Med., 1940, 72, 479.
15. Wintrobe, M. M., Clinical Hematology. Ed. 2, Lea and Febiger, Philadelphia, 1946.
16. Greenberg, G. R., Ashenbrucker, H., Lauritsen, M., and Wintrobe, M. M., The anemia of infection. IV. The lack of relationship between the diversion of iron from the plasma in infection and the origin of the anemia. J. Clin. Invest., 1947, 26, 114.

THE ANEMIA OF INFECTION. IV. THE LACK OF RELATIONSHIP BETWEEN THE DIVERSION OF IRON FROM THE PLASMA AND THE ORIGIN OF THE ANEMIA[1]

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The pronounced and persistent hypoferremia which accompanies infection, and the rapidity with which intravenously injected iron is removed when given in such cases (1), have led to the suggestion that the anemia of chronic infection results from a local iron deficiency in the bone marrow. Since the quantity of free protoporphyrin in the erythrocytes has been found increased in association with the anemia of infection, the possibility exists that this anemia is the consequence of deficient formation of hemoglobin resulting from a lack of iron.

The studies to be described here were designed to test the validity of this hypothesis. Iron was infused continuously in patients with chronic infection in order to determine whether, by raising the iron level to the normal value, synthesis of hemoglobin could be induced. These observations have made it possible to study the rate and degree of diversion of iron from the plasma in infection. Several observations also have been made of the uptake of intravenously administered radioactive iron by the red cells of patients with acute and chronic infections. Evidence will be presented in this communication suggesting that while a rapid removal from the plasma of injected iron occurs in infection, another factor, rather than lack of iron, may be responsible for the development of anemia.

METHODS

Ferrous ascorbate was used for the continuous infusion studies. A 2 per cent aqueous solution was carried through a Seitz filter. With a syringe this was added to a bottle containing 500 ml. sterile 5 per cent glucose solution; thus, the final solution contained 4 mgm. of iron per 100 ml. Since ferrous ascorbate is a complex with varying amounts of iron (2), each lot had to be analyzed, and the amount used varied accordingly. To obtain quickly the elevated plasma iron level, a single dose of 10 mgm. of iron as the ascorbate was injected intravenously shortly after starting the infusion. Considerable difficulty was encountered because of the frequent development of phlebitis after prolonged infusion of iron. This may have been due to the fact that the solutions were too acid (the undiluted ferrous ascorbate solutions exhibited a pH of about 4), or it may have been caused by the continued exposure of the blood vessels to relatively high concentrations of iron. Later it was found that it is possible to raise the pH somewhat if an excess of ascorbic acid is added and the solution is titrated with alkali, as we have done in studies with radioactive iron (3). The radioactive iron for the uptake studies was prepared according to the procedure described in the preceding paper (3), but in patients pyrogen-free, water was used, and the final solution was heated at 70° C. for 3 hours.

The methods for preparing and electroplating Fe^{59} in the blood samples have been described elsewhere (4). In these studies, blood volume has been estimated by assuming a value of 80 ml. per kgm. body weight (5). A few determinations of hemin Fe^{59} have been made according to the technique described in the previous paper (3).

Plasma iron was estimated according to the method of Kitzes, Elvehjem and Shuette (6).

RESULTS

Diversion of intravenously injected iron:

In Figure 1 are presented the results of the continuous intravenous infusion of iron in patients with chronic infection and anemia. It will be seen that very great difficulty was experienced in maintaining an elevated plasma iron level in the presence of infection. In order to maintain the plasma concentration at the normal level, it was necessary to employ several times the quantity of iron that might have been expected on the basis of single intravenous injections of iron. When the quantity of iron infused per unit of time was doubled, the plasma iron level increased by much less than a factor of 2. On the other hand, it was relatively easy to produce a high plasma iron level in the iron-deficient patient shown in the same figure. It is known that the iron-deficient patient takes up iron rapidly for hemoglobin formation. Consequently the drain on the plasma iron in the pres-

[1] Aided by a grant for the study of the pathogenesis of the anemia of infection from the United States Public Health Service; and by grants for the study of hematology and nutrition from the Upjohn Company and Parke, Davis and Company.

ence of infection must be considerably greater than that associated with increased hemoglobin synthesis following the administration of iron in iron deficiency anemia.

That the forced elevation of the plasma iron level did not produce increased hemopoiesis is shown by the results in the case of C. S. (Figure 2). Although this patient's iron level was maintained at approximately the normal value for as much as 72 hours, and enough iron was infused in the 2 attempts to form 31 and 80 grams of hemoglobin, respectively, little increase in reticulocytes occurred, and no significant increase in hemoglobin per 100 ml. of blood took place.

Table I summarizes data presented in paper I of this series (1) on the rate of disappearance from the plasma of iron after a single intravenous injection. From these data it is possible to show that in both the normal subject and in the patient with infection, some mechanism acts to prevent the plasma iron level from rising to the expected values after the injection of iron. In the patient with

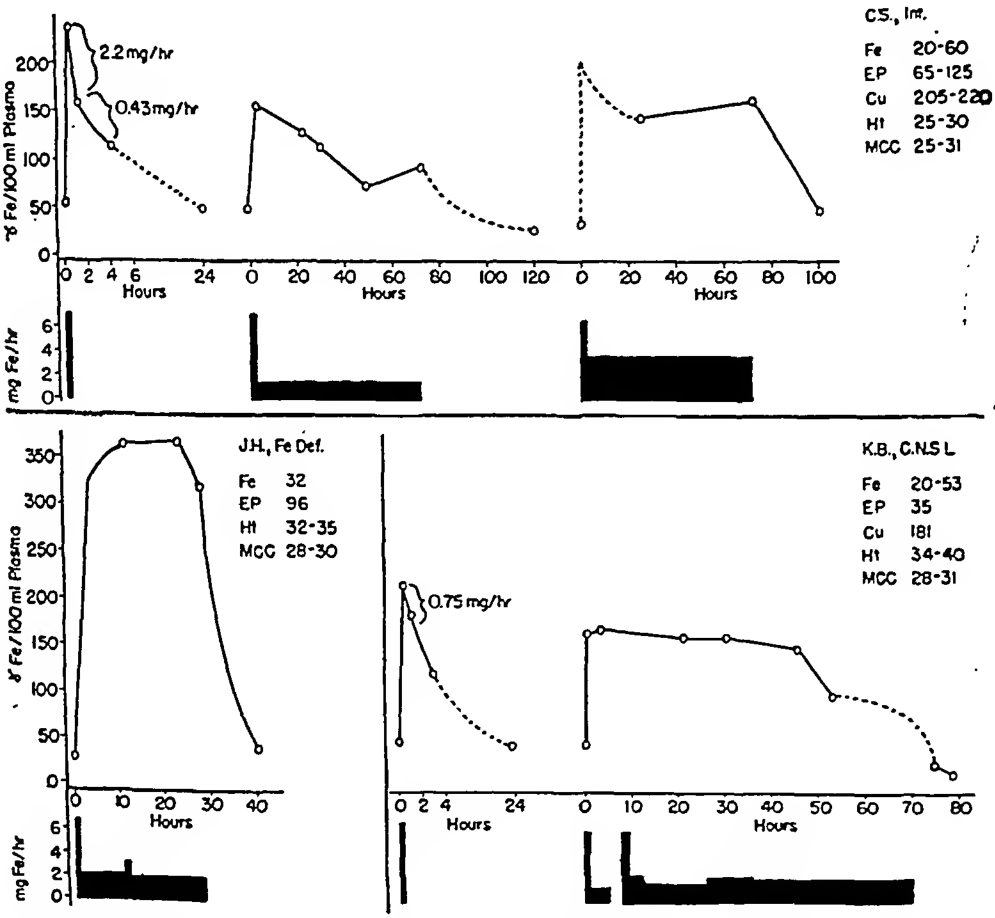

Fig. 1. The Effect on the Plasma Iron Content of Single and Continuous Injections of Iron Ascorbate in a Patient with Anemia Associated with Infection (C.S.), in a Patient with Iron Deficiency Anemia (J.H.), and in a Patient with Central Nervous System Syphilis (K.B.)

Under each patient's initials are given the plasma iron (Fe), the erythrocyte protoporphyrin (E.P.), the serum copper (Cu), the volume of packed red cells (Ht.), and the mean corpuscular hemoglobin concentration (MCC). [For normal values see (1).]

Note that in the cases of infection the plasma iron content could not be maintained at a level higher than normal, even though much larger quantities of iron were being injected intravenously than would be expected to be necessary from the rate of disappearance of single injections of iron. This was not true in the case of iron deficiency anemia, even though the need for iron was great.

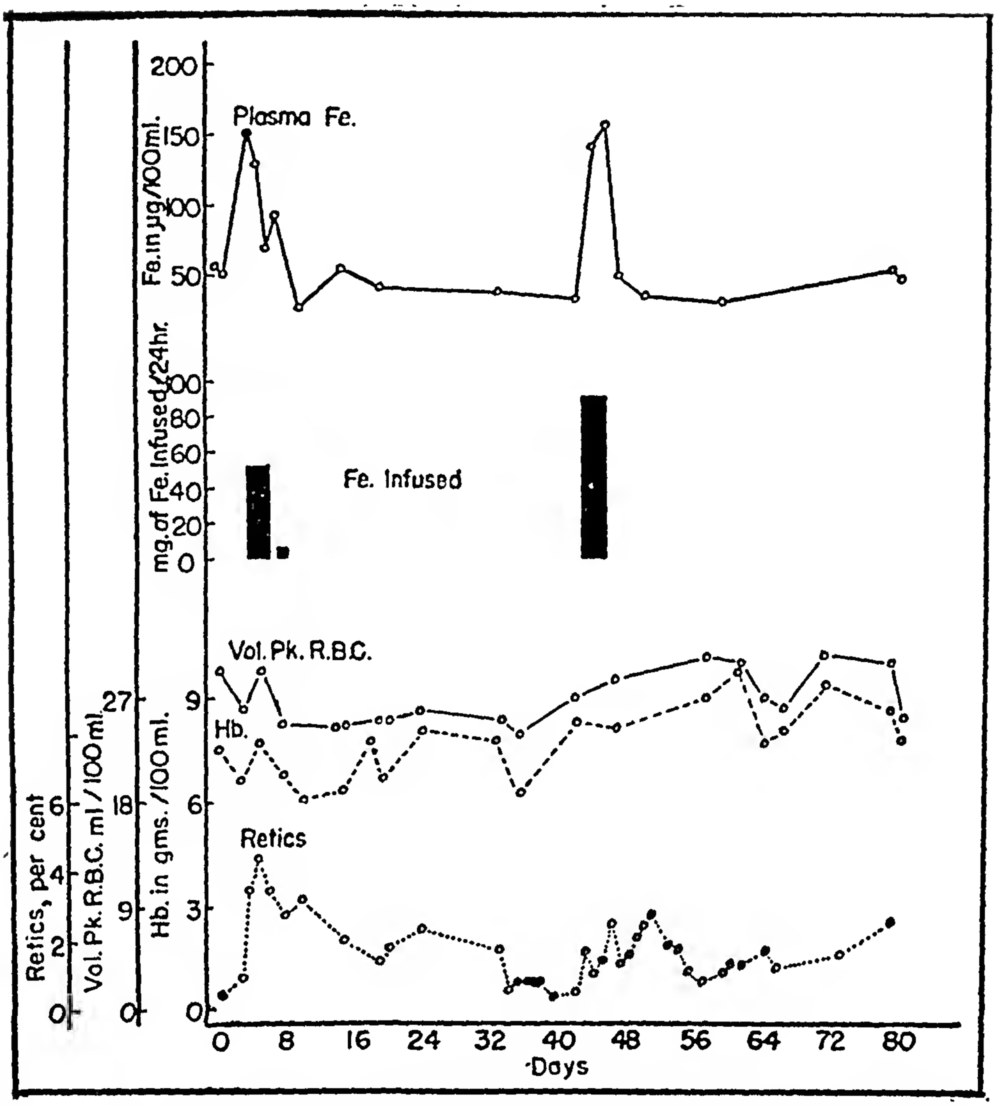

FIG. 2. FAILURE TO INDUCE BLOOD FORMATION OR TO PRODUCE A SIGNIFICANT RETICULOCYTE RESPONSE IN A PATIENT WITH A CHRONIC INFECTION, EVEN WHEN IRON WAS GIVEN CONTINUOUSLY BY VEIN AND THE PLASMA IRON WAS MAINTAINED AT A NORMAL LEVEL

Further details concerning this patient (C.S.) are presented in Figure 1.

infection this "brake" apparently acts at a lower level of iron. If it is assumed that the blood volume corresponds to 80 ml. per kgm. body weight, and that the patients and the normal subjects had average packed red cell volumes of 35 and 45 ml. per 100 ml., respectively, it is possible to calculate that, on the average, the plasma iron of the patients should have risen 250 µg. per 100 ml., and the plasma iron of the normal subjects should have increased 311 µg. per 100 ml. of plasma after the injection of 137 µg. and 130 µg. iron per kgm., respectively. Thus, in the patients, on the average, a maximum level of $43 + 250 = 293$ µg. per 100 ml. should have been attained, and in the normal subjects a peak value of $126 + 311 = 437$ µg. per 100 ml. should have been reached. The actu observed average levels were 196 and 315 µ respectively. Failure to observe higher values w not due to a failure to obtain the true peak, sin in one normal subject blood samples were drav each minute or two for 10 minutes after the i jection of iron; the peak rise was at approximate 5 minutes. Thus, the rate of loss of iron from t plasma in the first few minutes is approximate 100 times as rapid as in the succeeding hou The data indicate that the rate of disappearance iron from the plasma of a patient with infectio is about twice that seen in the normal subject the time following the initial few minutes aft the injection of iron (Table I).

TABLE I

Rate of disappearance of iron from the plasma following a single intravenous injection of iron in normal subjects and in patients with infection

	Fe injected	Initial plasma iron level	Maximum rise 5 minutes after injection	Iron lost per hour per kgm. At hour 1	At hour 2.5	At level of 140 µg. iron per 100 ml. plasma
Normal subjects						
Number of subjects	13	13	13	13	13	4
	µg. per kgm.	*µg. per 100 ml.*	*µg. per 100 ml.*	*µg.*	*µg.*	*µg.*
Range	137	85–237	262–392	−38 to +11	−4 to −25	−11 to −13
Average	137	126	315	−10	−12	−12
Patients with infections						
Number of subjects	13	14	14	14	14	10
	µg. per kgm.	*µg. per 100 ml.*	*µg. per 100 ml.*	*µg.*	*µg.*	*µg.*
Range	130	16–90	137–246	0 to −38	0 to −39	−15 to −90
Average	130	43	196	−17	−27	−27

In order to determine how rapidly the mechanism operating in infection can readjust to prevent a further rise in the plasma iron level, 3 injections of iron, 2 in a period of 1 hour, were given to a patient suffering from pneumonia. The results of this experiment are shown in Figure 3. It will be observed that the rise obtained after the third injection was only slightly higher than that obtained after the second injection given 1 hour before. On the basis of the peak rise after the second injection it might be expected that the level following the third injection would have been 334 µg. iron per 100 ml. The observed rise was to 266 µg. per 100 ml. Apparently the mechanism preventing the rise in plasma iron is able to readjust itself quickly in infection.

The uptake of Fe^{59}:

Figure 4 illustrates the results of preliminary studies on the rate of incorporation of intrave-

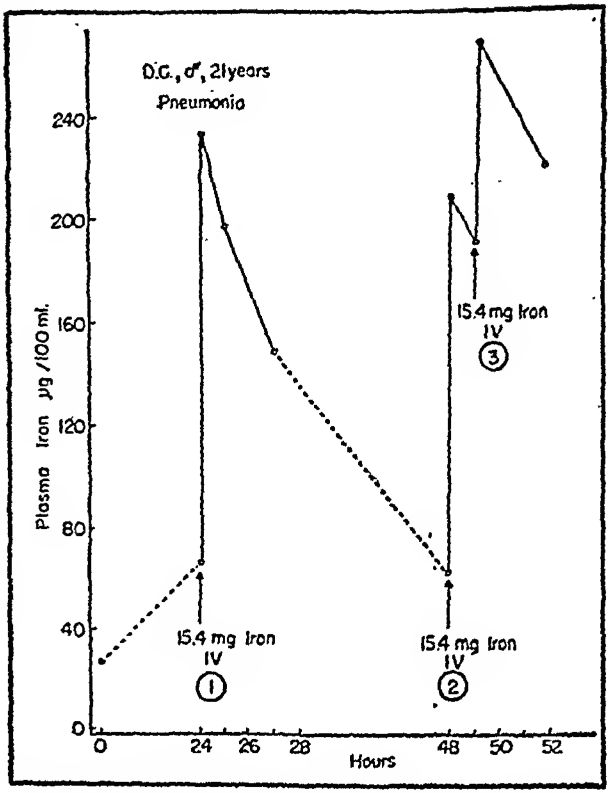

FIG. 3. THE EFFECT OF ATTEMPTS TO RAISE THE PLASMA IRON LEVEL BY THE INTRAVENOUS ADMINISTRATION OF REPEATED DOSES OF IRON IN THE PRESENCE OF INFECTION

It was difficult to maintain a high plasma iron level, even when a second injection was made before the plasma iron content had dropped. Note also that the rise following the third injection was relatively slight as compared with the rise following the previous injections. This suggests that a "braking" mechanism exists.

nously injected Fe^{59} into the erythrocytes as hemoglobin iron in patients with various infections, and in normal subjects. The dose of Fe^{59} employed in these studies ranged between 3 and 4 mgm., depending on the body weight. These data suggest that the degree of impairment of the uptake of Fe^{59} appears to be roughly proportional to the severity of the infection. It will be noticed that while there was an initial delay in the uptake of Fe^{59} by the patients with infection as compared to the normal subjects, in some of the patients after the first few days the rate of uptake approximated that of normal subjects, and the labelled iron was utilized almost completely. An example of this is seen in the case of T. G. who was suffering from a long-standing osteomyelitis. This pa-

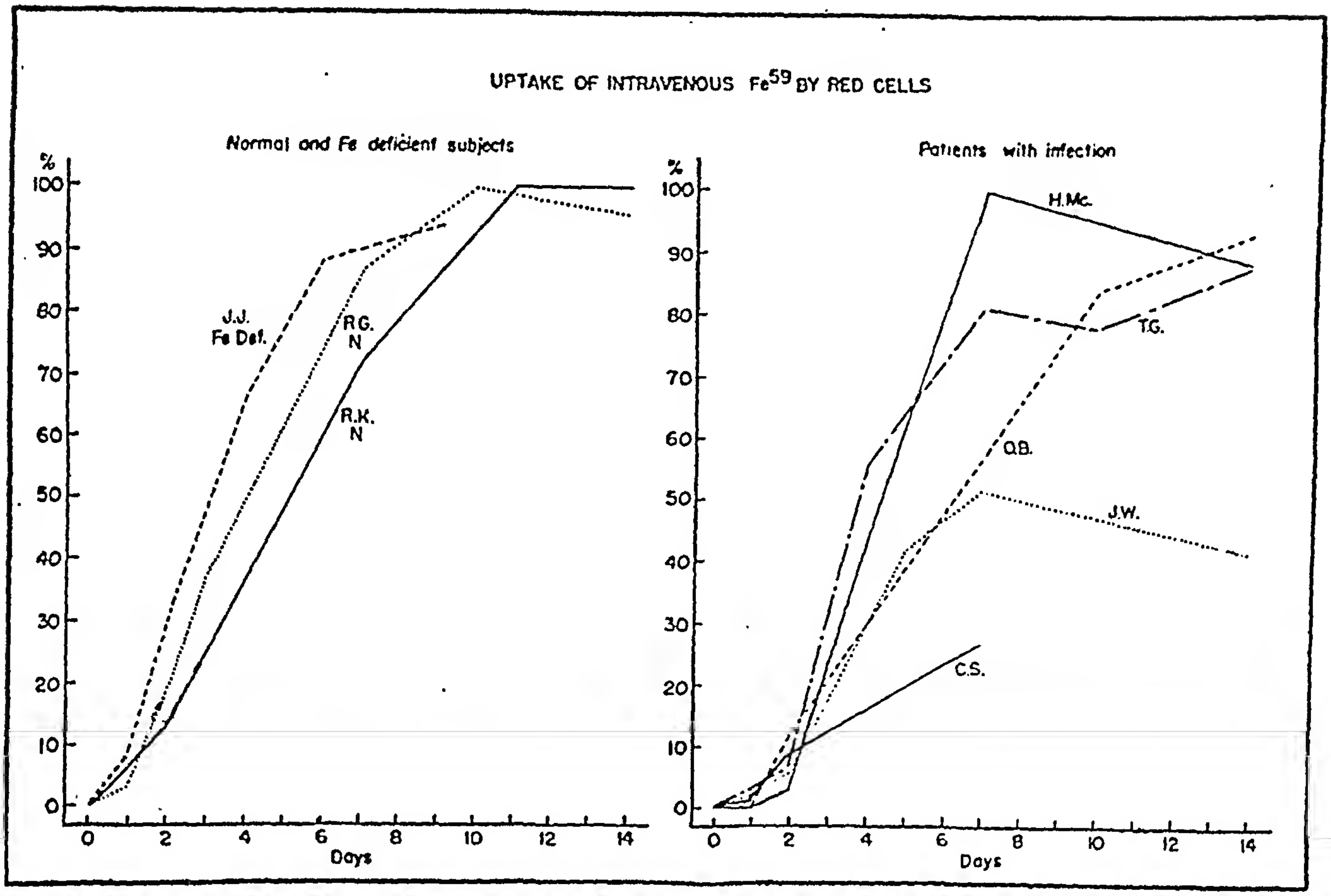

Fig. 4. Uptake of 3 to 5 mgm. Intravenously Injected Radioactive Iron (Fe^{59}) by the Red Cells of Normal (N) and Iron-Deficient (Fe Def.) Patients, and in Patients with Various Infections

In the patients with severe infections (C. S. and J. W.) there was not only a delay in uptake, but the uptake never was complete; whereas in those less ill, less difference was observed as compared with uptake in normal individuals. C. S. had chronic suppurative arthritis and chronic pyelonephritis. The volume of packed red cells (Ht.) was 28 ml. per 100 ml. blood. J. W. had subacute bacterial endocarditis, Ht. 30 ml. Both O. B. and T. G. had osteomyelitis and the volume of packed red cells was 39 and 40 ml., respectively. H. Mc. had rheumatic fever, Ht. 42 ml.

tient exhibited extremely low plasma iron concentration, rapid diversion from the plasma of intravenously injected iron, high erythrocyte protoporphyrin level and elevated serum copper, in addition to anemia. He was refractory to all forms of iron therapy (1). Yet approximately 100 per cent of the small dose of Fe^{59} which was given did enter his red cells. This was to be expected since, at a rate of hematopoiesis which maintains hemoglobin at 50 per cent of the normal, it can be calculated that about 12 mgm. of iron is being converted into hemoglobin in a man of average size (7).

Table II presents evidence that Fe^{59} appearing in the red cells after intravenous injection is present as hemoglobin iron. Hemin was isolated and its Fe^{59} content per mgm. of iron was compared with the Fe^{59} of the original blood per mgm. of hemoglobin iron. Such data have been presented for pigs in the preceding paper (3).

DISCUSSION

These observations indicate that in both normal subjects and in patients with infection, a mechanism operates which prevents the plasma iron level from increasing beyond certain limits. The major loss of injected iron occurs during the first few minutes when the rate of loss may be as much as 100 times as rapid as the loss in the ensuing hours. The initial loss of iron is approximately of the same absolute order of magnitude in both the normal subject and in the patient with infection, whereas the ensuing rate of loss is about twice as rapid in the presence of infection. This braking mechanism is so powerful that as more iron is given and the level is raised by repeated

TABLE II

Quantity of red cell Fe^{59} existing as hemoglobin iron

Subject	Status	Day following Fe^{59} injection	Percentage of RBC Fe^{59} present as hemoglobin
R.K.	Normal female	21	105*
R.G.	Normal male	16	107**
O.B.	Osteomyelitis	21	100*
A.G.	Pernicious anemia with iron deficiency	21	99*

* The actual quantity of isolated hemin analyzed for Fe^{59} was determined by measuring the iron content of an aliquot part (13) and calculating from the known percentage of Fe in hemin.

** The quantity of isolated hemin analyzed for Fe^{59} was determined by weighing after drying at 100° C.

injections or by continuous intravenous infusion of iron, it becomes increasingly difficult to maintain the plasma iron concentration. In a study of the use of intravenous iron tolerance curves for the differentiation of anemias, Waldenström (8) likewise concluded that a braking mechanism exists which prevents the attainment of calculated peak plasma iron values.

In spite of the existence of such a mechanism, it was possible to raise the plasma iron level temporarily to normal values in anemic patients suffering from chronic infections; yet hemoglobin formation did not take place. The strongest argument against the hypothesis that iron is the limiting factor in the anemia of chronic infection is the fact that both intensive and prolonged therapy with iron, administered orally and intravenously, will not bring about increased hemoglobin formation (1). Actually, iron therapy has been given the most severe test of any employed thus far. Schaefer (9) was able by intensive iron therapy to raise the serum iron level of children with infection to approximately normal values without effecting a change in the anemia. Brøchner-Mortensen and Stein (10) treated patients with tuberculosis with iron by mouth for several months at a time. These patients showed a greater and more constant rise in the serum iron than was observed in those given no iron, but the average change in hemoglobin before and after treatment was less than one per cent.

It cannot be maintained that a normal plasma iron level must exist before iron can be used for hematopoiesis, for in such a case, in the face of persistent hypoferremia, the anemia of infection should be progressive and ultimately should become very severe. Yet it is well known that the anemia tends to become fixed usually at a moderate and, often thereafter, at a rather stable level. Furthermore, in the presence of active hematopoiesis we have observed, like others (11), that in pernicious anemia after liver therapy as well as in iron deficiency anemia, the plasma iron level tends to remain low until the anemia has wholly or largely disappeared. Very active hematopoiesis can, thus, take place when the plasma iron content is low. Finally, as has been shown here by the use of radioactive iron, if small amounts of iron are given they can be utilized for the formation of hemoglobin in spite of the existence of infection. That this should be found to be the case is to be expected, since otherwise no red corpuscles containing hemoglobin would be available to replace those which become worn out in the course of time.

As a consequence, it must be concluded that although diversion of iron from the plasma occurs in infection, the anemia itself does not result from a lack of iron. Some other factor may be deficient, or some enzyme system may be interfered with by some process associated with infection. In agreement with Brøchner-Mortensen and Stein (10) it appears that there is a lack of capacity on the part of the bone marrow to utilize iron for hemoglobin synthesis rather than unavailability of iron.

In spite of the evidence that the lack of iron does not cause the anemia, it is clear that in acute and chronic infection a very marked diversion of iron from the plasma occurs. The system taking up intravenously injected iron in infection apparently withdraws it from the plasma with more avidity than an iron-deficient subject uses iron for hemoglobin formation. The site of diversion of the iron is the subject of the paper which follows (12).

SUMMARY

1. Patients with anemia of chronic infection have been subjected to continuous intravenous infusions of iron for periods as long as 72 hours.

2. Considerably more iron was required to maintain a normal plasma iron level than would be expected from the results of a single intravenous in-

jection of iron. The higher the plasma iron value desired, the more inefficient was the infusion.

3. In neither case did the infusion of iron result in increased hemoglobin formation.

4. Calculations have been made suggesting that a braking mechanism exists which prevents the plasma iron from rising above a certain level.

5. Evidence has been presented from radioactive iron studies and observations of the nature of the anemia, that some iron is capable of entering the erythrocytes as hemoglobin in the anemia of chronic infection.

6. Since even very intensive iron therapy is ineffective, it follows that iron is not the limiting factor in the production of the anemia, even though diversion of iron from the plasma does occur.

BIBLIOGRAPHY

1. Cartwright, G. E., Lauritsen, M. A., Humphreys, S. R., Jones, P. J., Merrill, I. M., and Wintrobe, M. M., The anemia of infection. I. Hypoferremia, hypercupremia and alterations in porphyrin metabolism in patients. J. Clin. Invest., 1946, 25, 65.
2. Friend, D. G., Iron ascorbate in the treatment of anemia. New England J. Med., 1938, 219, 910.
3. Wintrobe, M. M., Greenberg, G. R., Humphreys, S. R., Ashenbrucker, H., Worth, W., and Kramer, R., The anemia of infection. III. The uptake of radioactive iron in iron-deficient and in pyridoxine-deficient pigs before and after acute inflammation. J. Clin. Invest., 1947, 26, 103.
4. Greenberg, G. R., Humphreys, S. R., Ashenbrucker, H., Lauritsen, M., and Wintrobe, M. M., A simple apparatus and procedure for preparing and electroplating radioactive iron. Blood, 1947, 2, 94.
5. Moore, C. V., Dubach, R. Minnich, V., and Roberts, H. K., Absorption of ferrous and ferric radioactive iron by human subjects and by dogs. J. Clin. Invest., 1944, 23, 755.
6. Kitzes, G., Elvehjem, C. A., and Schuette, H. A., The determination of blood plasma iron. J. Biol. Chem., 1944, 155, 653.
7. Greenberg, G. R., and Wintrobe, M. M., A labile iron pool. J. Biol. Chem., 1946, 165, 397.
8. Waldenström, J., Jarnbelastningar och vad de Lära oss om Järnomsättningen, Om Järn och Järnterapi, Utgiven av ab Ferrosan, 1944.
9. Schaefer, K. H., Untersuchungen uber den exogenen Eisenstoffwechsel bei fieberhaften Infekten im Kindesalter. Klin. Wchnschr., 1940, 19, 979.
10. Brøchner-Mortensen, K., and Stein, K. S., Studies on the iron content of serum in patients with acute and chronic infections. Acta Tuberculosea Scandinav., 16, 334.
11. Moore, C. V., Doan, C. A., and Arrowsmith, W. R., The mechanism of iron transportation: Its significance in iron utilization in anemic states of varied etiology. J. Clin. Invest., 1937, 16; 627.
12. Greenberg, G. R., Ashenbrucker, H., Lauritsen, M., Worth, W., Humphreys, S. R., and Wintrobe, M. M., The anemia of infection. V. Fate of injected radioactive iron in the presence of inflammation. J. Clin. Invest., 1947, 26, 121.
13. Delory, G. E., Preparation and analysis for iron of hemin and hemoglobin. Analyst, 1943, 68, 5.

THE ANEMIA OF INFECTION. V. FATE OF INJECTED RADIOACTIVE IRON IN THE PRESENCE OF INFLAMMATION[1]

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Since hypoferremia is so consistently associated with the anemia of infection (1), and since injected iron appears to be diverted from the plasma in infection (1, 2), it seemed desirable to determine the fate of the diverted iron.

In this study, radioactive iron was injected intravenously into normal rats and in rats with acute inflammation. The blood and tissues were then analyzed after a period of time for radioactivity. Preliminary studies were made in which only the blood and liver were analyzed in order to determine the appropriate amount of iron to employ. Radioactive iron was also injected intravenously into dogs, and analyses were carried out on a number of tissues, including the exudates.

METHODS

The radioactive iron used in these studies was injected as ferric chloride in physiological saline. For the rats, the concentration was adjusted so that about 0.2 ml. was injected for each 100 grams body weight. In all the rats the same tuberculin syringe was employed for this purpose. In order to be certain that the solution did not enter subcutaneously, the vein on the lateral aspect of the tail was exposed under nembutal anesthesia and the solution was injected intravenously. In one experiment Fe^{59} was injected intraperitoneally.

Inflammation was produced by the intramuscular injection of 0.5 ml. of turpentine. Robscheit-Robbins and Whipple (3) have shown that the resulting sterile abscess can be compared with a bacterial abscess. In some animals, 0.5 ml. of a mixed culture of *S. aureus* and *E. coli* was injected intramuscularly. Injections of bacteria or turpentine were made about 2 hours before Fe^{59} was administered.

The methods for preparing and electroplating the Fe^{59} from the biological samples have been described elsewhere (4). Blood volume was estimated by assuming that the normal mammal possesses 80 ml. blood per kgm. body weight. Hemoglobin was determined in a representative sample of the tissue to be analyzed for radioactivity by a modification of the method of Greenberg and Erickson (5). The radioactivity attributable to the tissue hemoglobin was then subtracted from the total radioactivity of the tissue.

Blood samples were taken in the rats from the abdominal aorta. Blood hemoglobin determinations were made by the photoelectric oxyhemoglobin method of Bell, Chambers and Waddell (6).

The white rats used for these studies were virgin females obtained from the Sprague-Dawley Company and from the Carworth Farms. The dogs were mongrels secured locally.

RESULTS

Table I summarizes the results of analyses of the blood and liver of rats which received Fe^{59} intravenously. It will be seen that usually the rats which had been subjected to acute inflammation showed more Fe^{59} in the liver than was the case in the control animals. On the other hand, the control rats showed better uptake into the red cells than those with inflammation. In general, the sum of the radioactivity in the blood and liver of the

TABLE I

Distribution of injected (i.v.) Fe^{59} in rats 48 hours after injection

Number of rats	Experimental procedure	Amount Fe^{59} injected	Proportion found in: Blood	Liver	Blood plus liver
		γ per 100 grams	*per cent*	*per cent*	*per cent*
3	Turp. i.m. 1 ml.	10	15.2	59.5	74.8
3	None	10	35.2	36.3	71.5
4	Turp. i.m. 0.5 ml.	10	9.1	61.1	70.2
5	None	10	24.3	38.5	62.8
4	Turp. subcut. 0.5 ml. Bact. mix. 0.25 ml.	10	33.7	25.5	59.3
4	Bact. mix. i.m. 0.25 ml.	10	22.0	32.8	54.8
3	Turp. subcut. 0.5 ml.	10	39.7	22.0	61.7
4	Turp. subcut. 0.5 ml.	10	46.8	17.2	64.0
4	None	10	38.8	25.5	64.3
3	Turp. i.m. 0.5 ml.	3	26.1	37.8	63.9
3	None	3	39.1	24.6	63.7

[1] Aided by a grant for the study of the pathogenesis of the anemia of infection from the United States Public Health Service; and by grants for the study of hematology and nutrition from the Upjohn Company and Parke, Davis and Company.

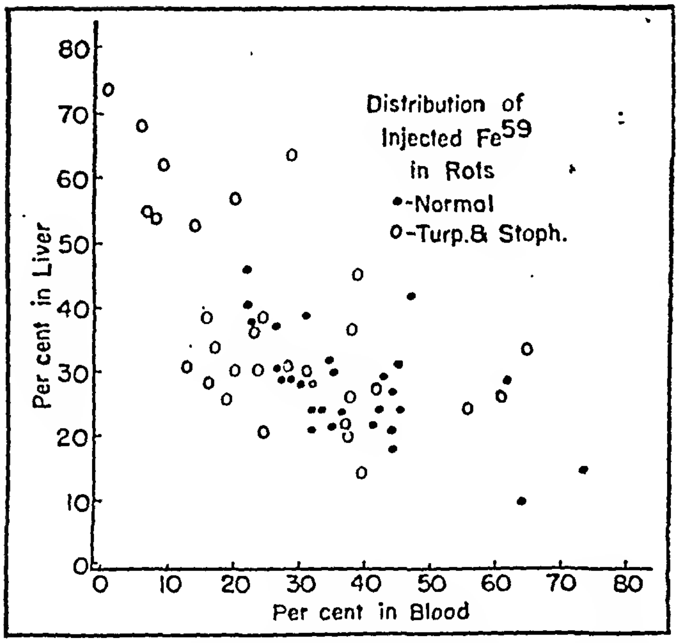

FIG. 1. TO ILLUSTRATE THE INVERSE CORRELATION OF THE QUANTITY OF RADIOACTIVE IRON IN THE BLOOD AND THAT IN THE LIVER FOLLOWING ITS INTRAVENOUS INJECTION BOTH IN NORMAL RATS AND IN RATS RECEIVING BACTERIAL OR TURPENTINE INJECTIONS

control animals was approximately equal to the same sum in the animals with inflammation. This is graphically illustrated in Figure 1.. In this figure the percentage of Fe^{59} in the liver of each animal is plotted against the corresponding Fe^{59} percentage in the blood. It may be observed that the points for the control animals and for those with inflammation fall along the same general line of correlation. When the iron was not found in the blood, it was in the liver. In no case, however, did the values for normal animals fall in the upper portion of the curve.

It should be pointed out that certain animals injected with turpentine apparently did not exhibit retardation of uptake of Fe^{59} by the red cells. The results in these animals together with their controls have been included in Figure 1. Sprague-Dawley rats were apparently considerably more sensitive to turpentine inflammation than were those of the Carworth Farm. Unfortunately, at the time these experiments were conducted it was very difficult to obtain the former animals. In these rats the liver and blood Fe^{59} percentages approached those of the normal animals. However, whether or not diversion to the liver occurred, the reciprocal relation of blood and liver Fe^{59} was maintained.

TABLE II

Distribution of Fe^{59} (25 µg. per 100 grams) injected intraperitoneally in rats

Rat no.	Experimental procedure	Time after injection	Proportion found in			
			Blood	Liver	Spleen	Total
		hours	*per cent*	*per cent*	*per cent*	*per cent*
1	Control	66	24.3	9.3	0.2	34
2	Control	66	18.7	6.4	0.8	28
3	Control	66	25.8	9.5	0.6	36
Average			22.9	8.4	0.5	32
4	Turp. i.m. 1 ml.	66	18.7	13.3	3.4	35
5	Turp. i.m. 1 ml.	66	15.7	9.7	1.1	26
6	Turp. i.m. 1 ml.	66	17.9	12.0	0.7	31
Average			17.1	11.7	1.7	31
7	Control	90	26.9	8.1	*	35**
8	Control	90	30.6	3.9	0	35
9	Control	90	28.5	*	0.7	35**
Average			28.6	6.0	0.4	35
10	Turp. i.m. 1 ml.	90	24.9	11.5	1.0	37
11	Turp. i.m. 1 ml.	90	19.8	9.3	*	29**
12	Turp. i.m. 1 ml.	90	17.5	10.8	0.7	29
13	Turp. i.m. 1 ml.	90	22.4	9.4	1.0	33
Average			21.1	10.3	0.9	32

* Sample lost.
** Calculating by assuming lost sample to be equivalent of average finding in other animals.

In Table II are shown the results of an experiment in which Fe^{59} was injected intraperitoneally, rather than intravenously, in rats. Analyses of the blood, liver and spleen were made on ½ the animals at approximately 3 days, and on the other ½ approximately 4 days following the injection of Fe^{59}. It will be seen that the distribution of the Fe^{59} tended to follow the same pattern as that observed after intravenous injection, except that in the latter case more was recovered in the period of time the animals were studied. It cannot be said with any assurance that an increase occured in the relative percentage of Fe^{59} in the spleen in the animals with inflammation as compared with their controls.

Table III presents an experiment in rats in which analyses were made of the Fe^{59} content of normal and inflamed muscles together with analyses of the radioactivity of the livers and spleens. As will be observed, very little Fe^{59} could be accounted for in either the inflamed or normal muscle 48 hours following the turpentine injection. On the other hand, the liver and blood again ac-

TABLE III

Distribution of injected (i.v.) Fe^{59} 10 μg. per 100 grams body weight

Rat no.	Experimental procedure	Proportion of injected Fe^{59} found in				
		Leg muscle (per gram wet weight)		Whole blood	Liver	Blood plus liver
		Normal leg	Inflamed leg			
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	Staph. E. coli } i.m.	0.6	1.1	28	31	59
2	Turp. i.m.	0.0	0.4	48	14	62
3	Turp. subcut.	0.0	4.4	38	21	59
4	Turp. Staph. E. coli } i.m.	1.0	2.0	31	30	61
5 to 8	None			39	25	64

counted for most of the Fe^{59}. Apparently a small increase in the proportion of Fe^{59} takes place in the inflamed muscle.

Table IV presents the results of an experiment in which Fe^{59} was injected into 2 dogs, one of which had received turpentine intramuscularly. The dose of iron employed was much larger than those used in the rat experiments. It will be seen that most of the Fe^{59} was found in the liver even in the normal dog. This is in agreement with the results of Hahn and coworkers (7) who noted that the major portion of a large dose of intravenously injected Fe^{59} is deposited in the liver, mainly as storage iron. It will be noticed that the percentage of Fe^{59} in the blood of the normal animal was more than twice that in the blood of the animal with inflammation. In addition it will be observed that more Fe^{59} was found in the spleen of the dog with inflammation than in the normal animal. The spleen is said not to enter into iron metabolism to the degree that the liver is concerned (8). It is of interest, finally, that no significant quantity of Fe^{59} was found in the inflamed muscle, in the exudate of the sterile abscess, or in the excreta. It should be mentioned that the analyses of the stools and bone marrows were made difficult by the presence of inorganic salts. The results are to be regarded only as approximate indices of the proportion of Fe^{59} carried to the stool and the bone marrow.

DISCUSSION

These results indicate that in infection a significant proportion of injected radioactive iron is not diverted away from the usual organs engaged in iron metabolism. Since the liver is the major iron-storing organ it is to be expected that most of the iron would be found there. It is of interest that Sandberg, Gross and Holly (9, 10) analyzed the spleen and liver from human autopsy specimens in chronic disease accompanied by anemia, and in various types of cancer with and without associated anemia. They found large accumulations of iron and copper in the liver and spleen, even when anemia was absent; and, in the presence of anemia, in amounts out of proportion to the degree of ane-

TABLE IV

Distribution of injected (i.v.) Fe^{59} in dogs

Experimental procedure and amt. Fe^{59} given		Proportion of injected Fe^{59} found in							
		Blood	Liver	Spleen	Plasma	Muscle per gram dry wt.	Stool and urine	Marrow per gram dry wt.	Total
day		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	No turp.								
	Fe^{59} 0.44 mgm. per kgm.								
2	Fe^{59} 0.44 mgm. per kgm.								
3	Sacrificed	5.89	86.70	2.04	0.20	0.0002	0.10	0.004	95
1	Turp. 10 ml. i.m.								
	Fe^{59} 0.31 mgm. per kgm.								
2	Fe^{59} 0.31 mgm. per kgm.								
3	Sacrificed	2.30	81.30	4.40	0.04	normal 0.0006 inflamed 0.0040	0.05	0.004	88

The inflammatory exudate in an animal given turpentine contained 0.1 per cent of the injected Fe^{59} per 100 ml.

mia. The present studies indicate that while more iron is found in the liver and spleen in infection, the increase in these tissues apparently corresponds to the quantity of iron not used for hemoglobin formation as a result of the retarded hemopoiesis. As has been shown in the preceding paper (2), the diversion which occurs, while very real, apparently is not the primary cause of the anemia of infection.

The greater quantity of iron found in the inflamed tissues as compared with that in the normal tissues was not of such an amount as to account for the persistent hypoferremia which accompanies infection. Menkin (11) found an accumulation of intravenously administered iron in the inflammatory tissues of rabbits. However, although his figures indicate that an average of 67 per cent more iron was present in the inflamed tissue after iron was injected than before, quantitatively the total amount of iron concerned was very small. He injected about 10 mgm. of iron and found 6.5 mgm. per 100 grams of dry inflamed tissue. From his description it seems unlikely, however, that more than 2 grams of dry tissue was inflamed; thus only 0.13 mgm. could be accounted for in this way. This represents but 1.3 per cent of the 10 mgm. of iron injected. This is of the same order of magnitude as was found in our studies.

It might be argued that this quantity of iron, if removed continuously, could produce hypoferremia in a patient with chronic infection. It does not seem plausible, however, that a comparatively small area of inflammation could take up sufficient iron to produce significant hypoferremia and impair hemoglobin synthesis. The data which have been presented suggest that it is the diversion of iron to the liver and spleen which is correlated with the hypoferremia.

With further reference to the fate of plasma iron diverted in the presence of inflammation, it may be pointed out that Hahn *et al* (12) showed that the urine and bile contain negligible quantities of iron injected intravenously in doses even larger than those used in the present study. In balance studies, Schaefer (13) found no evidence of increased excretion of iron in children with infection. It may be noted also that in a previous paper of this series (14), an iron-deficient pig with infection was described which was given large therapeutic doses of iron intravenously. This animal apparently did not excrete a significant quantity of iron, since on relief of the infection all of the injected iron could be accounted for as hemoglobin.

Our data offer no evidence as to the reason for the diversion of the iron from the plasma to the liver and, to a much smaller extent, to other tissues. It must be assumed that this diversion of iron is intimately related to some process related to infection. The present studies indicate that the lack of iron is not the fundamental factor in the pathogenesis of the anemia of infection. The presence of excess erythrocyte protoporphyrin and serum copper in cases of infection associated with anemia (1) suggests that no limitation exists in these substances. By elimination, the protein moiety of hemoglobin may be suspected as being involved. This view is tenable, furthermore, because it is well known that a disturbance in protein metabolism occurs in infections. It has been shown that in acute infections the urine nitrogen is very greatly increased (15), the same being true, though to a lesser extent, in chronic infections. It is generally recognized that the plasma albumin concentration is an index of protein reserves (16). Since globin is derived from these reserves (17), the fact that in chronic infection the plasma albumin concentration may be low (18) is probably significant in relation to the pathogenesis of the anemia of infection.

SUMMARY

1. Radioactive iron was injected intravenously in normal rats and dogs, and in animals with acute inflammation, in order to determine the fate of the iron.

2. A number of rats with inflammation showed retarded hemopoiesis and increased deposition of Fe^{59} in the liver and spleen as compared to the control animals.

3. There was good inverse correlation between the Fe^{59} in the liver and in the blood in both the normal rats and in the animals with inflammation.

4. Analyses of the inflamed tissues for Fe^{59} indicated that no significant quantity of iron is diverted to the inflamed area.

5. Insignificant percentages of large doses of intravenously injected iron were found in the exudate of a sterile abscess and in the excreta of a dog that had received turpentine intramuscularly.

6. It has been concluded that the major diversion of plasma iron in infection is to the ordinary storage tissues, mainly the liver.

7. This diversion is related to the hypoferremia.

BIBLIOGRAPHY

1. Cartwright, G. E., Lauritsen, M. A., Jones, P. J., Merrill, I. M., and Wintrobe, M. M., The anemia of infection. I. Hypoferremia, hypercupremia, and alterations in porphyrin metabolism in patients. J. Clin. Invest., 1946, 25, 65.
2. Greenberg, G. R., Ashenbrucker, H., Lauritsen, M., and Wintrobe, M. M., The anemia of infection. IV. The lack of relationship between the diversion of iron from the plasma and the origin of the anemia. J. Clin. Invest., 1947, 26, 114.
3. Robscheit-Robbins, F. S., and Whipple, G. H., Infection and intoxication. Their influence upon hemoglobin production in experimental anemia. J. Exper. Med., 1936, 63, 767.
4. Greenberg, G. R., Humphreys, S. R., Ashenbrucker, H., Lauritsen, M., and Wintrobe, M. M., A simple apparatus and procedure for preparing and electroplating radioactive iron. Blood, 1947, 2, 94.
5. Greenberg, A., and Erickson, D., A method for the quantitative determination of hemoglobin in tissues. J. Biol. Chem., 1944, 156, 679.
6. Bell, G. H., Chambers, J. W., and Waddell, M. B. R., The routine estimation of haemoglobin as oxyhaemoglobin. Biochem. J., 1945, 39, 60.
7. Hahn, P. F., Granick, S., Bale, W. F., and Michaelis, L., Ferritin. VI. Conversion of inorganic and hemoglobin iron into ferritin iron in the animal body. Storage function of ferritin iron as shown by radioactive and magnetic measurements. J. Biol. Chem., 1943, 150, 407.
8. Hahn, P. F., The metabolism of iron. Medicine, 1937, 16, 249.
9. Sandberg, M., Gross, H., and Holly, O. M., Changes in retention of copper and iron in liver and spleen in chronic diseases accompanied by secondary anemia. Arch. Path., 1942, 33, 834.
10. Gross, H., Sandberg, M., and Holly, O. M., Changes in copper and iron retention in chronic diseases accompanied by secondary anemia. II. Changes in liver, spleen and stomach. Am. J. M. Sc., 1942, 204, 201.
11. Menkin, V. J., Studies on inflammation; fixation of metal in inflamed areas. J. Exper. Med., 1930, 51, 879.
12. Hahn, P. F., Bale, W. F., Hettig, R. A., Kamen, M. D., and Whipple, G. H., Radioactive iron and its excretion in urine, bile and feces. J. Exper. Med., 1939, 70, 443.
13. Schaefer, K. H., Zur Pathogenese der Infektanämie, insbesondere ihre Beziehungen zum Eisenstoffwechsel des wachsenden Organismus. Klin. Wchnschr., 1940, 19, 590.
14. Wintrobe, M. M., Greenberg, G. R., Humphreys, S. R., Ashenbrucker, H., Worth, W., and Kramer, R., The anemia of infection. III. The uptake of radioactive iron in iron-deficient and in pyridoxine-deficient pigs before and after acute inflammation. J. Clin. Invest., 1947, 26, 103.
15. Grossman, C. M., Sappington, T. S., Burrows, B. A., Lavietes, P. H., and Peters, J. P., Nitrogen metabolism in acute infections. J. Clin. Invest., 1945, 24, 523.
16. Whipple, G. H., and Madden, S. C., Hemoglobin, plasma protein and cell protein. Their interchange and construction in emergencies. Medicine, 1944, 23, 215.
17. Daft, F. S., Robscheit-Robbins, F. S., and Whipple, G. H., Abscess nitrogen metabolism in anemic and non-anemic dog. Reserve stores of protein apparently involved. J. Biol. Chem., 1937, 121, 45.
18. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry, Vol. I. Williams and Wilkins, Baltimore, 1937, p. 693.

THE CIRCULATING RED CELL AND PLASMA VOLUME AND THE DISTRIBUTION OF BLOOD IN LARGE AND MINUTE VESSELS IN EXPERIMENTAL SHOCK IN DOGS, MEASURED BY RADIOACTIVE ISOTOPES OF IRON AND IODINE[1]

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During World War I a recently developed dye method of measuring plasma volume (1) was employed, to a limited extent, in the investigation of hemorrhagic and traumatic shock in wounded soldiers. Those studies (2 to 4) showed that shock was associated with varying degrees of reduction in the measurable total blood volume. It was assumed that this reduction resulted in a decreased venous return to the heart, which accounted for diminished blood flow and death in vascular collapse. It was also felt that severity of shock as judged clinically was proportional to the degree of blood volume reduction. Cannon (5) summarized the results of these investigations in 1923. There was little change in this conception of the fundamentals of shock for several years, and, as might be expected, little improvement in the therapy of shock.

Blalock (6) concluded that the decrease in blood pressure in experimental traumatic shock was due to loss of blood into damaged areas, or to hemorrhage into the intestinal tract (7). Moon (8) on the basis of pathological observations on dogs dying in prolonged shock (peritonitis) concluded that increase in capillary permeability permitting the leakage of plasma from the vascular bed was chiefly responsible for loss in blood volume.

As improvements in the original dye method of measuring plasma volume became available (9 to 11), attention was again concentrated on blood volume in shock. It was shown (12) that surgical operations may be accompanied by a loss of plasma and red cells greater than can be accounted for by measured blood loss. A reduction in blood volume severe enough to produce severe peripheral vascular collapse was found to be brought about by the excessive sweating accompanying artificially induced fever (13). It was observed for the first time that shock produced in dogs by thermal burns was *preceded* by great reductions in circulating plasma volume (14, 15).

Hemoconcentration of blood in large vessels has been associated with shock, and was thought to be characteristic. Scudder (16) suggested that the determination of the specific gravity of whole blood and plasma might be useful as a guide to therapy.

Interest in the hemodynamics of shock came into a renewed focus during the early years of World War II. An extensive historical review of our knowledge of shock was published by Harkins in 1941 (17) and a briefer summary of the known physiology of shock by Wiggers in 1942 (18).

The many blood volume studies in experimental shock in animals (19) and of traumatic shock in man (20 to 22) carried out during the war have amply confirmed the early conception that severe oligemia is a constant characteristic of the condition.

That significant leakage of plasma from capillaries whose permeability is increased in shock does not occur, has been clearly demonstrated by

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Fine and Seligman (23 to 25). The opinion has been expressed by Noble and Gregersen that the reduction in blood volume is due to loss of whole blood at the site of injury (22). Since their subjects were accident cases, and the extent of blood loss suffered could be calculated only on the basis of blood volume measured after recovery, these observations do not indicate whether or not the entire reduction of blood volume as measured in shock could be accounted for by loss of blood into the various sites of injury.

Early in the course of our work we noted that the circulating red cell volume, as measured by the specific method of injecting red cells tagged with radioactive iron, was always some 15 per cent less than when measured by the dye plasma hematocrit method (26). This led us to question to some extent the reported changes in circulating red cell volume obtained by the latter method. Furthermore, our early studies in hemorrhagic shock in dogs, in which there was no evidence at autopsy of internal bleeding, consistently showed a reduction in *circulating* red cell volume greater than the cell volume removed, whereas dogs bled only moderately, and in whom shock symptoms did not occur, did not exhibit such a deficit in circulating red cells. This deficit was termed "trapping."

Root (27) and Chambers (28) made microscopic observations of capillary circulation in the mesentery of rats and dogs. Both observers noted that after severe hemorrhage, intestinal manipulation, and occlusion of blood flow in the leg, numerous plexi of capillaries contained closely packed erythrocytes at a complete standstill, the cells in circulation flowing through the larger arteriovenous shunts. This suggested that some portion of the red cells found to be "trapped" in our hemorrhaged dogs might still be within the vascular bed, and yet be temporarily isolated from the main course of the circulating blood. It was of interest to know where in the capillaries of the various organs such "trapping" might occur, and to what extent it might progress in relation to the clinical signs of impending, early or late shock.

Methods previously described (29) had enabled us to measure the quantity of red cells and plasma within the minute vessels of the organs in relation to the total circulating red cell and plasma volume. We concluded from these studies that (*1*) all erythrocytes were in active circulation at all times; (*2*) 17 per cent of the total blood volume was within the capillaries; (*3*) there are pronounced gradients in the hematocrit of whole blood as it flows from larger to smaller vessels; and (*4*) that there are no significant reserves of red cells in the body of the normal dog.

The purpose of the experiments herein reported was to (*1*) measure reductions in red cells and plasma volume in a variety of experimental shock conditions, (*2*) determine the degree of "trapping" of red cells in shock, (*3*) locate if possible the site of red cell trapping, and (*4*) determine the extent of disturbance in normal capillary flow in early, reversible and irreversible shock.

METHODS

Plasma volume was determined by the method of Gibson and Evelyn (30), red cell volume by the method of Gibson and Peacock (31). Intravascular measurements of hemoglobin (25) and of radioactive iodine (25) were made as described by Fine and Seligman.

MATERIAL STUDIED

Shock was experimentally induced in normal healthy (stray) dogs by hemorrhage, muscle trauma with and without hemorrhage (Blalock crusher) (32), muscle ligation (33), occlusion of blood flow in extremities (tourniquets) (34), burns (flame (14) and hot water) and toxins (Shiga (35) and Clostridium (36)). Morphine narcosis and, in a few cases, nembutal anesthesia were used. Clinical condition of animals was followed by changes in mean arterial pressure, obtained by cannulation of a femoral or carotid artery, registered with mercury manometer.

Four groups of experiments were completed, here classified by the type of blood volume measurements carried out.

Series I. Total plasma (dye) and circulating red cell volumes (radio-iron) in the normal state, and during periods of lowered arterial pressure (shock), were determined. Some of these animals received plasma or albumin therapeutically.

Series II. In addition to the observations made in Series I, the quantity of total (by hemoglobin or Fe^{59} measurements) and circulating (by Fe^{59}) red cells within the minute vessels of several organs was determined.

Series III. In addition to the observations made in Series I and II, the quantity of circulating plasma (by radio-iodo-protein) within the minute vessels was determined.

Series IV. Initial circulating cell and plasma volume was determined when the animals were in shock induced by tourniquets applied to both hind legs for 9 hours. These animals were treated with varying quantities of bovine albumin in 5 per cent and 25 per cent solution. A

repeat cell and a plasma volume were determined at varying intervals after the albumin infusion.

Series I. Gross volume studies.

Determinations were carried out in dogs in whom shock was experimentally induced as follows:

- Hemorrhage:
  - Controls—Bled for samples only—2 dogs.
  - Moderate bleeding but not to shock level—1 dog.
  - Severe bleeding to shock levels—10 dogs.
  - Severely bled, treated with dog plasma—2 dogs.
  - Severely bled, treated with bovine albumin—1 dog.
  - Severely bled, treated with whole dog blood transfusion—1 dog.
- Muscle trauma:
  - Blalock crusher—3 dogs.
  - Ligation of both gastrocnemii—1 dog.
  - Bilateral occlusion tourniquets—1 dog.
- Burns:
  - Flame, treated with bovine albumin—3 dogs.
  - Water (98° F.)—1 dog.
- Bacterial toxins:[2]
  - Shiga toxin—2 dogs.
  - Clostridium toxin—1 dog.

In all of these experiments, the plasma and red cell volumes were determined prior to the experimental procedure. The total quantity of blood withdrawn, in both sampling and intentional and accidental hemorrhage, and the hematocrit of that blood were measured. The net loss of plasma and cells was taken as the quantities of plasma and cells removed minus the quantities of plasma and tagged red cells given (as whole blood) for radio-iron cell volume measurements. The difference between initial and net loss of plasma and cells was termed the "expected volume." Repeat volume measurements, termed "found," were made in shock. If the found cell or plasma volume was less than the expected values, these discrepancies were termed "trapped"; if greater than the expected values, they were termed "mobilized." The quantity of trapped cells of plasma was expressed as a percentage of the expected volumes. Auricular, arterial, or venous hematocrits were measured directly. The average body hematocrit was calculated from total circulating plasma volume and red cell volume as measured by radio-iron.

Series II.

In 5 dogs the total and circulating red cells (but not plasma) within the minute vessels were measured, as described above. In addition, circulating red cell volume was measured in the normal and later in the shocked state.

Two animals were bled about ⅓ and ½ of their blood volume, and died in shock. One dog had both gastrocnemii ligated, was also bled and died in shock. Two dogs received Shiga toxin, and had moderate hemorrhages. Both were sacrificed, one before the onset of peripheral collapse and the other in deep shock.

[2] Experiments in collaboration with Freedberg, Haimovici and Blumgart (35).

Series III.

In 5 morphinized dogs, the total and circulating red cells, and the circulating plasma (by radio-iodo-protein) were measured, as described above, in addition to measurements of total circulating red cells and plasma in the normal and later in the shocked state.

Two animals were bled, one (25–128) moderately, and one (25–116) severely. Both were sacrificed before the onset of extreme peripheral collapse. Dog 25–129 was bled as previously described (37), a mean arterial pressure of below 30 mm. Hg being maintained for 3 hours; all of his own blood was then returned, and a large transfusion was given (800 ml.). The initial red cell and plasma volumes were determined *after* the transfusion. The blood transfusion was of no benefit, and the animal was sacrificed terminally. Dog 25–130 had both hind legs burned with water at 98° F. and was sacrificed *in extremis*. Occlusion tourniquets were applied to both hind legs of dog 25–132 for 9 hours, and the animal was in deep shock when sacrificed.

Series IV.

A series of 16 morphinized dogs, in whom deep shock was produced by the application of tourniquets to both legs for 9 hours, was treated with intravenous bovine albumin solutions (34). All of these animals had marked hemoconcentration, the hematocrits all being above 60, and mean arterial pressure fell to below 70 mm. Hg shortly after the release of the tourniquets. The auricular hematocrit was greatly lowered by the albumin, whether given in 5 per cent solution or 25 per cent solution, with or without additional fluid or salt. Circulating red cell volume was determined before and after the administration of albumin.

RESULTS

Series I.

The extent of trapping or mobilization of red cells or plasma, expressed as percentages of the expected final volume, found in all animals in this series is summarized in Table I.

In the control dogs, bled less than 10 per cent of total blood volume for sampling only, no trapping of cells or plasma was observed.

Of 11 dogs in hemorrhagic shock, bled a net of from 27 to 50 per cent of initial total blood volume, 10 showed trapping of red cells of from 4 to 48 per cent of the expected volume; 9 dogs showed trapping of plasma of from 8 to 43 per cent. One dog, excessively hydrated, showed mobilization of both red cells and plasma, and 1 animal treated with bovine albumin solution showed mobilization of plasma. The dog treated with a large whole blood transfusion (25–129) showed trapping of both red cells and plasma.

TABLE I

Trapping of red cells (by radio-iron and dye methods) and of plasma (dye method) in experimental shock in dogs

Experiment	Procedure	Percentage trapped		Percentage mobilized	
		Vrr*	Vpd*	Vrr	Vpd
135–9	Control		11	1	
25–42	Control	12	3		
	After plasma	10	7		
25–128	Hemorrhage	4	20		
25–41	Hemorrhage	37	20		
	After plasma	11	32		
25–45	Hemorrhage	18	19		
	After plasma	22	28		
135–7	Hemorrhage	8	43		
135–8	Hemorrhage	15	40		
25–104	Hemorrhage—dehydrated	17	9		
25–105	Hemorrhage—hydrated			63	86
25–118	Hemorrhage	48	9		
25–120	Hemorrhage	11	8		
25–129	Hemorrhage—whole blood	4	7		
25–119	Hemorrhage—albumin	23			24
135–11	Blalock crusher	9	9		
135–15	Blalock crusher	21	24		
135–16	Blalock crusher			22	6
135–90	Ligation of muscles	12	24		
25–132	Tourniquets	10	57		
131–39	Burn—albumin	32	24		
131–40	Burn—albumin	15			42
131–41	Burn—albumin	19			86
25–130	Burn	16	15		
SA–2**	Shiga toxin	3			16
SA–3**	Shiga toxin	44	26		
135–136	Clostridium toxin	20			10

* Vrr = Red cell volume by radio-iron method.
Vpd = Plasma volume by dye method.
** Experiments in collaboration with Freedberg *et al* (35).

Two animals injured with the Blalock crusher showed trapping, and 1 showed mobilization of cells and plasma. Trapping of both cells and plasma also was found in a dog both of whose gastrocnemii were ligated, and in a dog in whom the blood flow to both hind legs had been occluded by tourniquets. All 4 burned dogs had extensive trapping of red cells, and 2 had trapping of plasma. Three of these, burned by flame, received bovine albumin, and 2 of these mobilized plasma.

Minimal trapping of red cells with mobilization of plasma was observed in 1 dog (SA–2) receiving Shiga toxin (not in shock) but another dog in severe shock showed extensive trapping of whole blood. The animal receiving Clostridium toxin trapped cells, but mobilized plasma.

Series II.

The unit quantities (ml. per gram) of total and circulating red cells in the minute vessels of the organs, and the ratio of circulating to total cells, are shown in Table II . Also shown are the percentages of expected total red cell volume represented by total and circulating red cells in the minute vessels of the entire organ masses analyzed. In addition, the quantity of trapped red cells found by gross volume measurements and the quantities found trapped in the minute vessels of the organ samples analyzed are shown.

With certain minor exceptions, the findings in the animal sacrificed while not in shock (SA–2) were at the lower limit of the normal range. The quantity of total and circulating red cells in the spleen was about ⅔ the normal average, but within the normal range; and in the kidneys, about ⅓ of normal. The percentage of expected red cell volume found as total and circulating cells in all the minute vessels was about normal. Trapping of cells by gross volume measurements was within the accepted limit of error, and no trapping was found in the minute vessels, the ratio f2 being 1.00.

In all 4 dogs in shock, the red cell content of the spleen, both total and circulating, was below normal, and only about ⅓ to ⅔ of this reduced content of cells was in active circulation. The spleen had been emptied of about ⅓ of its normal red cell content.

Total red cell values were higher than normal in dog SA–3 (Shiga toxin), lower than normal in dog 25–120 (severe hemorrhage), but showed no marked deviation from normal in the other 2 dogs. The values for circulating red cells were definitely below normal (except in SA–3, in which they were high), and in virtually all instances were lower than the total cell values.

In all 4 dogs, the ratio of red cells actively circulating to those contained in the entire minute vessel bed was less than 1, ranging from 0.76 to 0.83, averaging 0.80. In only one instance out of 26 was the f2 value for an individual organ greater than one (1.03). Thus about ⅕ of the total cells in the minute vesels were not in measurable circulation.

Series III.

The total and circulating red cell content, and circulating plasma and whole blood content of all the organs are given in Table III. The ratio (f2) of rapidly circulating to total red cells for indi-

TABLE II

Total and circulating red cell content of minute vessels in 1 dog not in shock and in 4 dogs in deep shock, in relation to expected and found total circulating red cell volume, and the ratio of actively circulating to total red cells in the minute vessels (f2)

Organ	Normal values		25–118 Hemorrhage—death in shock			25–120 Hemorrhage—albumin—death in shock			135–90 Ligation of gastrocnemii, with hemorrhage—death in shock			SA-2** Shiga toxin—sacrificed—not in shock			SA-3** Shiga toxin—sacrificed—in deep shock		
			Red cells		f2	Red cells		f2	Red cells		f2	Red cells		f2	Red cells		f2
	Range	Average	Total	Circulating		Total	Circulating		Total	Circulating		Total	Circulating		Total	Circulating	
	ml. per gram	*ml. per gram*	*ml. per gram*	*ml. per gram*		*ml. per gram*	*ml. per gram*		*ml. per gram*	*ml. per gram*		*ml. per gram*	*ml. per gram*		*ml. per gram*	*ml. per gram*	
Spleen	.18 to .56	.367	.146	.085	0.58	.138	.075	0.54	.097	.080	0.85	.292	.252	0.86	.218	.057	0.26
Liver	.02 to .08	.048	.060	.055	0.92	.025	.023	0.92	.044	.037	0.85	.058	.069	1.19	.070	.062	0.88
Lungs	.02 to .09	.063	.063	.055	0.88	.037	.038	1.03	.089	.081	0.90	.046	.047	1.01	.103	.090	0.89
Kidneys	.02 to .06	.037	.034	.028	0.82	.018	.016	0.89	.035	.030	0.93	.009	.012	1.23	.049	.045	0.91
Heart	.01 to .05	.022	.012	.007	0.58	.014	.012	0.85	.036	.024	0.57	.019	.017	0.91	.022	.020	0.85
Bowel	.004 to .015	.006	.006	.006	0.97	.003	.002	0.66	.006	.002	0.34	.012	.010	0.83	.007	.004	0.51
Muscle	.002 to .007	.004	.002	.001	0.59	.003						.007	.006	0.83	.007	.006	0.85
Average					0.78			0.83			0.74			0.98			0.73
Cells in minute vessels in ml.			58.9	47.2	0.80	28.3*	23.5*	0.83	54.6	44.6	0.82	52.8	52.8	1.00	40.0	31.2	0.76
Percentage of expected red cells in minute vessels		17	10.4	8.4		8.2	6.8		10.9	8.9		15.3	15.3		14.8	11.2	
Percentage of initial red cells bled			40			50			22			10			14		
Cells trapped by gross volumes			272			36			59			4			121		
Cells trapped by tissue analysis			12			5			10			0			9		
Percentage of expected red cell volume trapped in minute vessels			2.0			1.4			2.0			0			2.3		
Auricular hematocrit Initial			47.7			41.4			48.0			42.5			52.3		
Auricular hematocrit Final			41.8			38.6			51.5			38.6			58.7		

* Excluding muscle.
** Experiments in collaboration with Freedberg *et al* (35).

vidual organs, and for the entire organ masses analyzed; the percentage of expected as contrasted to circulating total red cell, plasma and whole blood volume found in the minute vessels; and the hematocrits of whole blood in minute and large vessels and auricle are also shown.

Changes in total red cell and whole blood content of the spleens of the 4 animals in whom ob-

TABLE III

Total and circulating red cell content, and circulating plasma and whole blood content of minute vessels in 5 dogs in experimental shock, in relation to expected and found total circulating red cell, plasma and whole blood volumes, and the ratios of actively circulating to total red cells in the minute vessels

Organ	Average normal values				25–128 Hemorrhage—sacrificed not in shock					
	Red cells	Plasma	Circ. whole blood	Hct.	Red cells: Total	Red cells: Circ.	Plasma	Circ. whole blood	Hct. of circ. blood	f2
				per cent						
Spleen	.367	.065	.420	82	.149	.099	.068	.167	59	0.66
Liver	.048	.115	.200	41	.029	.028	.101	.129	21	0.94
Lungs	.063	.115	.195	33	.052	.051	.104	.151	33	0.96
Kidneys	.037	.174	.205	15	.017	.016	.084	.104	17	1.04
Heart	.022	.049	.066	22	.005	.005	.041	.046	11	1.00
Bowel	.006	.050	.060	17	.004	.005	.019	.024	18	1.09
Muscle	.004	.014	.018	21	.003	.003	.008	.011	26	1.12
Brain	.006	.010	.013	18	.003	.004	.006	.010	40	1.20
Injured muscles										
Average				32					25.6	1.00
Excluding splenic blood				23*					21.1	1.05
Percentage of volume removed					34		23	30		
Total content in small vessels in ml.					29.9	30.8	87.9	118.6	25.2	1.03
Percentage of expected volume in small vessels	17	20	17		14.3	14.7	16.8	16.2		
Percentage of circulating volume in small vessels	17	20	17			15.4	17.2	16.7		
Red cells trapped: By gross volumes	0	0	0		9					
Red cells trapped: By tissue analysis	0	0	0		+1					
Percentage of expected cell volume trapped in small vessels					43					
Auricular hematocrit: Initial									39.2	
Auricular hematocrit: Final									35.8	

* Excluding splenic blood.

TABLE III—*Continued*

Organ	25–116 Hemorrhage—sacrificed early in shock						25–129 Hemorrhage—blood returned—Large transfusion—sacrificed in shock*					
	Red cells: Total	Red cells: Circ.	Plasma	Circ. whole blood	Hct. of circ. blood	f2	Red cells: Total	Red cells: Circ.	Plasma	Circ. whole blood	Hct. of circ. blood	f2
Spleen	.047	.054	.101	.155	35	0.15	.140	.105	.108	.213	48	0.75
Liver	.027	.019	.133	.152	12	0.71	.180	.158	.146	.304	52	0.88
Lungs	.040	.045	.136	.181	25	1.12	.089	.105	.124	.229	46	1.18
Kidneys	.029	.024	.138	.162	16	0.83	.048	.034	.167	.201	17	0.71
Heart	.004	.007	.038	.045	16	1.74	.028	.024	.039	.063	38	0.86
Bowel	.002	.003	.036	.039	8	1.44	.033	.030	.033	.063	30	0.91
Muscle	.003	.002	.010	.012	16	0.61	.011	.005	.008	.013	38	0.45
Brain							.009	.005	.005	.010	52	0.57
Injured muscles												
Average					16.0	1.08					40.1	0.78
Excluding splenic blood					15.2	0.92					44.5	0.80
Percentage of volume removed	49		37	44			0		0	0		
Total content in small vessels in ml.	39.4	32.1	172.1	204.1	15.7	0.82	133.2	98.4	122.0	220.4	44.6	0.74
Percentage of expected volume in small vessels	11.6†	9.5	32.5	23.4			21.6	15.9	21.4	18.6		
Percentage of circulating volume in small vessels	13.9	11.3	26.1	21.3			22.6	16.7	23.0	19.7		
Red cells trapped: By gross volumes	57						25					
Red cells trapped: By tissue analysis	7						35					
Percentage of expected cell volume trapped in small vessels	2.0						5.7					
Auricular hematocrit: Initial					43.1						60.2	
Auricular hematocrit: Final					33.4						62.6	

* Initial Vrr and Vpd determined *after* transfusion.
† Initial Vrr estimated from Vrpd × 0.85.

TABLE III—*Continued*

Organ	25-130 Burn both hind legs—sacrificed in deep shock (spleen not analyzed)						25-132 Tourniquets both hind legs—sacrificed in deep shock					
	Red cells		Plasma	Circ. whole blood	Hct. of circ. blood	f2	Red cells		Plasma	Circ. whole blood	Hct. of circ. blood	f2
	Total	Circ.					Total	Circ.				
Spleen							.083	.039	.073	.112	34	0.47
Liver	.048	.017	.112	.129	13	0.35	.046	.032	.081	.113	28	0.67
Lungs	.069	.052	.127	.179	29	0.75	.084	.081	.118	.199	41	0.99
Kidneys		.025	.144	.169	14		.058	.053	.157	.210	25	0.91
Heart	.021	.014	.045	.059	23	0.67	.029	.027	.035	.062	44	0.93
Bowel							.009	.010	.018	.028	35	1.05
Muscle	.009	.005	.010	.015	32	0.52	.008	.008	.007	.015	55	1.13
Brain	.006	.005	.010	.015	26	0.78	.009	.007	.005	.012	54	0.81
Injured muscles	.018	.005	.013	.018	26	0.26	.032	.011	.013	.024	50	0.34
Average					23	0.60					42	0.87
Excluding splenic blood											43	0.92
Percentage of volume removed	+3*	+2*	+2*				2		2	2		
Total content in small vessels in ml.	73.1	30.0	99.9†	129.9†	23.2	0.41	105.6	82.8	112.8	195.6	42.3	0.79
Percentage of expected volume in small vessels	16.4	6.7	15.2	11.8			16.6	13.1	12.5	12.5		
Percentage of circulating volume in small vessels	19.5	8.1	18.1	13.3			18.2	14.3	29.6	20.4		
Red cells trapped: By gross volumes	72						55					
Red cells trapped: By tissue analysis	43						23					
Percentage of expected cell volume trapped in small vessels	9.7						8.7					
Auricular hematocrit: Initial					48.6						49.1	
Auricular hematocrit: Final					48.4						69.3	

* Quantity of blood given for volume determination exceeded amount lost in sampling.
† Excluding spleen and bowel.

servations were made were striking. The total cell content was reduced by from 60 to 80 per cent, the plasma content remaining unchanged. Here again, as in Series II, the spleen had emptied about 20 per cent of the normal *total* minute vessel red cell content into the blood stream.

In both the untreated bled dogs, the total red cell content of minute vessels of all organs was well below normal. The lowest values were observed in the most severely bled animal, in whom circulating red cell contents were still lower.

Plasma content values were consistently about ⅔ of normal in all organs of the dog not in shock, but in the dog in early shock values for lung, liver and kidney were higher than normal, being elsewhere about normal. Whole blood values for all organs were below normal in both animals.

The animal receiving the large transfusion had higher than average normal total cell values in all organs, and higher than normal circulating cell values in liver, lungs and kidneys. Plasma values were at essentially normal levels. Whole blood values were above normal in liver and lungs, but otherwise were about normal.

The burned animal showed practically no deviation from average normal total cell content in the organs analyzed, but all values for circulating cells were low (no data were obtained on spleen, kidneys and bowel). Plasma values were on the low side, and whole blood values reflected the diminution in circulating cells and plasma.

In the animal with shock produced by tourniquets, total cell content values of all organs were consistently high, the circulating cell values approximating the normal range. Plasma values were low, to such an extent that the whole blood values were slightly below normal in spite of the high cell contents.

In this series, in 7 out of 28 instances f2 ratios greater than 1.0 were obtained. The weighted f2 value was 1.03 in the dog not in circulatory collapse, but in the 4 shocked animals values ranged from 0.60 to 0.82, averaging 0.74. In this series, as in Series II, about 20 per cent of the total cells within the minute vessels were not in active circulation.

Series IV.

In this series less than 10 per cent of the circulating red cell volume found in shock was removed in net sampling. In every case, the auricular hematocrit, markedly elevated after removal of tourniquets, was considerably lowered following the administration of albumin. The circulating red cell volume after albumin was higher than the expected volume (volume in shock less net loss in

sampling) in 10 dogs, showed no change in 4 dogs, and was lower in 2 dogs. Of the 10 showing an increase, the change was less than 10 per cent in 4, between 5 and 10 in 4, and over 10 per cent in 2 dogs.

Eight dogs survived more than 24 hours, after the removal of tourniquets (later to die of infection or be sacrificed), and 8 died within 24 hours. All of the 8 survivors showed increases over expected cell volume, but 2 that showed increases died. None of the animals that died showed an increase over expected volume, and 2 had decreases after therapy. Changes in circulating red cell volume, auricular hematocrit and mean arterial pressure are given in Table IV.

DISCUSSION

Virtually all of the animals in Series I, in which only gross red cell and plasma volume measurements were made, had reductions in total blood volume. In addition, they showed varying degrees of trapping of both red cells and plasma.

Considered by etiology of shock, the animals given bacterial toxins showed approximately 50 per cent trapping of red cells; those burned, 20 per cent; those whose muscles were injured, 15 per cent; those with severe or fatal hemorrhage, 25 per cent; and those whose blood loss was treated (without avail) with whole blood or protein solutions, 15 per cent. The majority of these animals underwent severe reductions in mean arterial and pulse pressure, and blood flow was markedly slowed, but, as far as the clinical condition of the animal could be judged by these observations, there was no obvious relationship between the degree of trapping and mean arterial pressure levels.

Some bled animals underwent hemodilution, as evidenced by a fall in auricular hematocrit, and some hemoconcentration, with a rise in auricular hematocrit. The burned animals (treated with albumin, and untreated) showed no marked change in hematocrit; those whose muscles were damaged showed considerable hemoconcentration, while those receiving toxins showed variable changes in hematocrit. There was no consistent relationship between the direction of, or degree of, change in hematocrit and the percentage of total red cell volume found to be trapped.

All of these animals underwent varying degrees of net loss of whole blood due to intentional bleeding or sampling. The findings cited above, however, indicate that characteristically, in shock,

TABLE IV

Changes in auricular hematocrit, mean arterial pressure and circulating red cell volume after treatment of shock induced by bilateral tourniquets with albumin solutions

Exp. no.	Wght.	Albumin i.v.	Hematocrit		Red cell volume			Change in cell volume		Change in m.a.p.	Result
			Before	After	Before	Expected	After	+	−		
	kgm.	*ml.*	*per cent*	*per cent*	*ml.*	*ml.*	*ml.*	*per cent*	*per cent*	*mm. Hg*	
2	12.7	500	77	45	530	480	510	5.7		+60	S
4	6.4	400	62	57	380	355	395	11.2		0	S
12	11.3	500	64	43	500	455	540	18.7		+10	S
13	18.2	615	69	39	760	700	760	8.6		+50	S
17	12.7	1,600	70	32	505	485	500	3.1		+50	D
19	13.2	1,000	67	32	550	485	495	2.1		+50	D
21	10.0	600	82	62	485	435	435	0		−40	D
6	7.8	80	85	57	445	450	450	0		−20	D
8	11.4	110	55	40	515	475	475	0		+50	D
27	7.7	75	77	56	370	340	335		1.3	−10	D
38	12.3	240	77	55	505	480	480	0		+20	D
41	9.1	75	67	48	315	295	220		25.4	−10	D
31	13.2	260	67	31	460	415	430	3.2		+40	S
33	7.7	155	65	34	250	220	230	4.5		+40	S
45	13.2	265	71	52	605	595	640	7.5		+45	S
47	26.4	500	62	40	1,080	1,060	1,105	5.2		+30	S

Dogs 2 to 21 inclusive received 5 per cent albumin in 0.85 per cent NaCl.
Dogs 6, 8, 27, 38 and 41 received 25 per cent albumin with no additional fluid.
Dogs 31 and 33 received 25 per cent albumin plus saline by stomach tube.
Dogs 45 and 47 received 25 per cent albumin plus 0.5 gram NaCl per 100 ml. plus water by stomach tube.
S = Survived 24 hours or more after release of tourniquets.
D = Died within 24 hours of release of tourniquets.

there is a *greater reduction in circulating cells and plasma than can be accounted for by known external blood loss.*

This discrepancy may be accounted for in 2 ways: (*1*) loss of cells and plasma from the vascular tree through internal bleeding into damaged areas, serous cavities or hollow viscera, and (*2*) isolation from the moving blood stream of cells and plasma that remain within some portion of the vascular tree. Evidence of the former phenomenon should be obtained at necropsy. Evidence of the latter phenomenon was found in the experiments in Series II and III.

There were 2 dogs in these series not in shock (SA-2 and 25-128), and they served as controls for the shocked animals. Other than reductions in blood volume due to known blood loss, neither dog showed any significant deviation from the normal. The percentage of total red cell, plasma and whole blood volume found within the minute vessels of individual organs, the ratio of rapidly circulating to total red cells in minute vessels, and the ratio of the hematocrit of blood in minute and large vessels to the auricular hematocrit were all within normal limits.

The shocked animals in these 2 series represent 1 case of early shock (25-116), 1 fatal hemorrhage (25-118), 2 cases of severe hemorrhage in which volume replacement with bovine serum albumin (25-120) and whole blood (25-129) was ineffective, 1 case of severe muscle trauma (135-90), and 1 case of arterial and venous occlusion (25-132), both untreated and fatal in degree, 1 fatal burn (25-130), and 1 case in which death was due to an overwhelming toxin (SA-3).

Certain findings were common to all of these animals, although varying in degree, regardless of the etiology of their peripheral vascular collapse. Without exception, the unit values (ml. per gram) for total red cells in the spleen were well below normal values. In the other organs, values were above normal in the transfused dog (25-129) and the animal dying of Shiga toxin (SA-3). High values were chiefly in lungs and liver and kidneys. Low values were consistently found in all 3 animals that were bled. No significant change from normal was found in the other animals.

Unit values for plasma were below normal in the tourniquet dog (25-132), about normal in the bled dog not in shock, but above normal in the

TABLE V

Percentages (net) of cells, plasma and whole blood removed by hemorrhage; the expected and found red cell, plasma and whole blood volumes; and the quantities of total and circulating red cells within the minute vessels

	Series II														
	25-118			25-120			135-90			SA-2*			SA-3*		
	Hemorrhage—death in shock			Hemorrhage—death in shock			Ligation of gastrocnemii—death in shock			Shiga toxin—not in shock			Shiga toxin—sacrificed in shock		
	C	P	W	C	P	W	C	P	W	C	P	W	C	P	W
Percentage of initial volume bled (net)	37	41	39	49	42	44	22	22	22	+2*	−1	−1	5	+1	+2
Percentage of initial volume expected	63	59	61	51	58	56	78	78	78	102	99	99	95	101	102
Percentage of initial volume found	42	55	44	46	54	51	68	59	63	102	116	110	54	75	64
Percentage of initial volume found in minute vessels — Total	6.6			4.2*			8.5			16.1			13.8		
Percentage of initial volume found in minute vessels — Circulating	5.3			3.4*			7.0			15.8			10.7		
Percentage of expected volume found in minute vessels — Total	10.4			8.1			12.5			16.1			14.5		
Percentage of expected volume found in minute vessels — Circulating	8.1			6.7			10.3			15.8			11.3		
Percentage of normal minute vessel volume* — Total	61			48			74			93			85		
Percentage of normal minute vessel volume* — Circulating	52			39			61			92			66		
Percentage of initial volume trapped — Whole body	21	4	17	5	4	5	10	19	15	+2	+16	+10	11	26	18
Percentage of initial volume trapped — Minute vessels	1.3			0.6			0.9			0			0.3		
Percentage of initial volume unaccounted for	19.7	4	17	4.4	4	5	9.1	19	15	+2	+16	+10	10.7	26	18
*Normal percentage of total volume in minute vessels: red cells 17, plasma 20, whole blood 17. (See Table I)	Final Vrr in dying animal. Determined value may be falsely high due to incomplete mixing of tagged red cells.			*Red cell content of minute vessels did not include muscle.			Evidence of large loss of cells and plasma into muscles at necropsy.			*Red cells given for Vrr exceeded net loss of cells in sampling. *Experiments in collaboration with Freedberg *et al.* (35)			110 ml. of bloody fluid found in gut at necropsy (19 per cent of initial whole blood volume).		

TABLE V—*Continued*

	Series III														
	25–116			25–128			25–129			25–130			25–132		
	Hemorrhage—sacrificed in early shock			Hemorrhage—not in shock			Hemorrhage—large transfusion—sacrificed in deep shock			Burn both hind legs—sacrificed in deep shock			Tourniquets both hind legs—sacrificed in deep shock		
	C	P	W	C	P	W	C	P	W	C	P	W	C	P	W
Percentage of initial volume bled (net)	41*	41	41	34	28	30	0*	0*	0*	+3*	+2*	+2*	−2	−2	−2
Percentage of initial volume expected	59	59	59	66	72	70	100	100	100	103	102	102	98	98	98
Percentage of initial volume found	49	78	66	66	71	68	96	93	95	86	86	86	89	42	60
Percentage of initial volume found in minute vessels: Total	6.8			9.4			21.6			16.8			16.3		
Percentage of initial volume found in minute vessels: Circulating	5.5	19.1	13.8	9.7	12.1	11.4	15.9	21.2	18.6	6.9	15.5	11.0	5.4	12.3	12.3
Percentage of expected volume found in minute vessels: Total	11.5			14.2			21.6			16.3			16.8		
Percentage of expected volume found in minute vessels: Circulating	9.3	24.5	20.9	14.7	17.1	16.3	15.9	21.2	18.6	6.7	15.2	10.8	5.5	17.5	12.6
Percentage of normal minute vessel volume*: Total	68			84			127			96			99		
Percentage of normal minute vessel volume*: Circulating	54	122	123	87	86	96	94	106	109	39	76	64	32	63	74
Percentage of initial volume trapped: Whole body	10	+19	+7	4.3	1	2	4	7	5	17	16	16	9	56	38
Percentage of initial volume trapped: Minute vessels	1.2			0			5			10			4		
Percentage of initial volume unaccounted for	8.8			0	1	2		7	6	7	16	16	5	56	38
*Normal percentage of total volume in minute vessels: red cells 17, plasma 20, whole blood 17. (See Table I)	*Initial red cell volume estimated at Vrpd × 0.85 Hemodilution: Hau fell from 43 to 33						*Initial Vrr and Vpd determined *after* transfusion. Radioactive blood given equalled amount bled in sampling.			*Radioactive blood given for volume determinations exceeded amount lost in sampling. Extensive edema both legs—blood tinged—could account for discrepancy.			Extreme swelling both hind legs; could account for enormous loss of plasma and probably of red cells		

burned dog (24–130), and in both the dog in early hemorrhagic shock (25–116) and the transfused dog (25–129). In all animals in shock, the unit value of circulating red cells tended to be less than that of total red cells. In both series, a total of 69 organ samples were analyzed for total and circulating red cells. In only 10 instances was the ratio (f2) of rapidly circulating to total red cells greater than unity. The weighted f2 for all organs was less than 1 in all 8 animals dying or sacrificed in shock. These values ranged from 0.60 to 0.83, averaging 0.76. The lowest ratio occurred in the burned dog, the highest in the bled dog in early shock and the bled dog treated with bovine albumin. The values for the other 5 dogs were from 0.73 to 0.79.

Thus from ⅓ to ⅕ of the red cells contained in the minute vessels throughout the body were not in active measurable circulation. This phenomenon was widespread, occurred in all the organs analyzed, and was not confined to sites of traumatic injury. The lowest f2 ratios were found in the muscles of the burned dog and the animal subject to occlusion tourniquets.

Since the techniques employed measure only red cells and plasma in active circulation, the findings reflect the state of blood flow through the minute vessel beds of the organs. It is apparent that in these shocked animals, the blood flow through all of the organs was reduced by from 20 to 40 per cent.

Thus another characteristic of shock is that, *regardless of etiology, there is a widespread stagnation of red cells within the minute vessels of all of the organs in the body.* The degree of this stagnation may be such as to indicate *a reduction in blood flow through minute vessels to from 60 to 80 per cent of normal.*

In Table V are listed for Series II and III (as percentages of initial volumes), the net amount of cells, plasma and whole blood removed by hemorrhage and sampling; the expected and found volumes; and the quantity of red cells, both total and actively circulating, measured as being within the vessels of the organs analyzed. These minute vessel red cell quantities are also expressed as percentages of the expected cell volume, and these in turn, as percentages of the fraction of total red cell volume normally found in circulation in the minute vessels. (See Table I for average normal values.) The percentage of expected cells, plasma and whole blood volume found trapped in the

vascular bed (by gross volume measurements) and in the minute vessels (by tissue analyses) are also shown. Finally, the red cells, plasma and whole blood not accounted for by measured trapping within minute vessels, are expressed as percentages of both initial and expected volumes.

The plasma content of minute vessels was not determined in Series II.

Dog SA-2 experienced a negligible net loss of whole blood (a slight net gain in red cells), and the fraction of total red cells found in the minute vessels was almost normal, 93 per cent. Only 2 per cent of gross trapping of red cells was found, and all of the cells in the minute vessels were in active circulation.

In both the dogs that were severely bled (25–118 and 120), the found volume of cells and plasma was less than expected. The fraction of the expected total red cell volume, found as total cells in the minute vessels, was below normal to a degree about equal to the net red cell loss, but the fraction found in active circulation was far lower than the percentage of cells lost. In both animals the gross volume trapping was far greater than that measured by organ analysis.

Dog SA-3 succumbed to the toxin and was in deep shock. Net blood loss was little, but the found cell and plasma volume was much less than expected. The minute vessels contained only 85 and 66 per cent of the expected total and circulating cell volume. Only a fraction of the total trapping was found in the minute vessels. The gut contained a large quantity of bloody fluid at necropsy.

Dog 135–90 experienced moderate blood loss in addition to trauma, and again the found red cell and plasma volume was considerably lower than the expected. The reduction from normal in fraction of expected total cells in the minute vessels was greater than the percentage of initial red cell volume removed, and the reduction of circulating cells was even greater. Again, gross trapping far exceeded that measured by organ analysis. There was evidence of a large loss of red cells and plasma into the the ligated muscles at necropsy.

Thus, all 4 dogs in shock experienced a greater reduction in total circulating cell volume than could be accounted for by net blood loss. In addition the proportion of total cells, both expected and found, in the minute vessels was less than normal, and the proportion in active circulation considerably less than normal.

In dog 25–118, 21.0 per cent of the initial cell volume was unaccounted for, but only 1.3 per cent was accounted for by measured trapping in minute vessels. Since the tagged cells for final red cell volume were given with the animal in a dying state, they may not have become completely mixed, and the final total red cell volume calculation may therefore have been falsely low.

In Series III (Table III), dog 25–128, not in shock, showed no gross or minute vessel trapping of either cells or plasma, and the found volumes equalled the expected volumes, but the percentages of total and circulating cells and circulating plasma in the minute vessels were about 15 per cent below normal. There was slight gross trapping but none by organ analysis.

Dog 25–116, in early shock, showed reduction in red cell volume greater than accounted for by hemorrhage; and reductions in the normal fraction of total and circulating cells in minute vessels. Considerable plasma had been mobilized so that, although total whole blood volume was reduced, the fraction of whole blood in minute vessels was higher than normal. There was evidence of slight trapping in the minute vessels, and of 10 per cent gross trapping.

None of the other 3 dogs experienced any net blood loss. In the animal transfused, 25–129, the found blood volume was about equal to the expected; the quantity of total cells and of plasma in the minute vessels was above normal, and the quantity of circulating cells in minute vessels only slightly below normal. Five per cent of the initial cell volume was found trapped in the minute vessels, by tissue analysis, and 4 per cent by gross volume measurements. The quantity of trapped cells was 27 per cent of the total cells in the minute vessels. About 7 per cent of the expected plasma volume was unaccounted for.

Similar but more severe changes were found in the tourniquet dog (25–132). Found circulating cell volume was 90 per cent of expected; plasma volume, 42 per cent; and whole blood volume, 60 per cent of expected. The portion of total cells in the minute vessels was normal, but only 32 per cent of the normal fraction was in active circulation. The minute vessels contained only 63 per cent of the normal complement of circulating

plasma, and as a result only 74 per cent of the normal quantity of whole blood was flowing through the minute vessels. Five per cent of the initial cell volume and 56 and 38 per cent of the initial plasma and whole blood volume were unaccounted for.

Thus in only 2 of the above experiments (25–118 and 120) was the amount of trapped cells unaccounted for by organ analysis greater than could be explained by whole blood loss into damaged areas or intestinal hemorrhage.

The actual quantities of red cells trapped within the minute vessels is a very small fraction of both the expected and found red cell volumes. *These quantities of trapped red cells, however, represent a very large percentage of the total cells within the minute vessels.*

The quantity of all the cells and plasma in the body, as determined by gross plasma (dye) and circulating red cell (radio-iron) measurements; and of the cells and plasma in the minute vessels (by analyses of tissue samples for radioactive iron and radioactive iodine) were measured for dogs in Series III. The arithmetic difference between whole body and minute vessel blood may be termed "large vessel blood." These radioactivity data permit of calculating the hematocrits of (*1*) the blood in minute vessels of individual organs, and hence the average weighted hematocrit of all the blood in the minute vessels of the organ analyzed, (*2*) the hematocrit of all the blood in the body, and (*3*) the hematocrit of that portion of blood contained only in the large vessels. These values are shown in Table VI, in comparison with corresponding values found in 5 normal dogs. They serve to bring out another characteristic disturbance in the circulation in shock, illustrated in Figure 1. The upper left-hand figure represents the normal percentage, in terms of degrees, of total blood volume contained within the large and minute vessels, the hematocrit of the blood being indicated by the cross-hatched inner circles. The other figures represent the degree of change from the normal relationship in the 5 dogs in Series III. In each case the entire circle represents the *final* found total blood volume; the larger angular section, the percentage of whole blood in the larger vessels; and the smaller section, the percentage of whole blood in the minute vessels. The cross-hatched sections again represent the hematocrit of the blood in the 2 compartments. The black sections in the centers of the circles represent the percentage of total minute vessel cells trapped out of circulation. Thus the graphs portray changes in distribution of cells and plasma within the vascular bed.

Dog 25–128 was not in shock; the percentage of final total blood volume in the minute vessels was normal, and the hematocrit of that blood was

TABLE VI

Hematocrits of blood in right auricle, large vessels, the whole body, and minute vessels; and the ratio of these hematocrits to the auricular hematocrit

	Normal dogs											
	25–131		25–127		25–114		25–113		25–111		Average	
	per cent	*R*	*per cent*	*R*	*per cent*	*R*	*per cent*	*R*	*per cent*	*R*	*per cent*	*R*
Auricular hematocrit	42.3	1.00	33.8	1.00	50.0	1.00	46.7	1.00	44.6	1.00	43.5	1.00
Large vessel hematocrit	40.7	0.96	31.3	0.93	47.3	0.95	45.2	0.97	32.3	0.73	39.4	0.90
Whole body hematocrit	40.0	0.95	32.9	0.97	44.3	0.88	45.3	0.97	31.9	0.71	38.9	0.90
Minute vessel hematocrit	37.6	0.89					35.8	0.77	24.2	0.54	32.5	0.75
Minute vessel hematocrit (excluding spleen)	26.3	0.62	25.9	0.77	16.5	0.33	24.6	0.53	22.8	0.51	23.4	0.54
	Dogs in experimental shock											
	25–128		25–116		25–129		25–130		25–132			
Auricular hematocrit	35.1	1.00	43.1	1.00	62.5	1.00	48.4	1.00	67.5	1.00	51.3	1.00
Large vessel hematocrit	28.5	0.81	32.2	0.75	54.0	0.86	38.1	0.79	65.0	0.96	43.6	0.85
Whole body hematocrit	28.1	0.80	28.8	0.67	52.3	0.84	36.1	0.75	60.5	0.89	41.2	0.80
Minute vessel hematocrit	25.9	0.74	15.8	0.37	44.4	0.71			43.2	0.64	32.3	0.63
Minute vessel hematocrit (excluding spleen)	25.0	0.71	15.2	0.35	44.4	0.71	23.1	0.48	43.2	0.64	30.2	0.59

only slightly elevated. The hematocrit of large vessel blood was definitely below normal. There was no trapping. Arterial hematocrit was 35.1.

Dog 25–116 was in early shock. The percentage of whole blood in the minute vessels was increased, the hematocrit of that blood was below normal, and there was slight trapping. The hematocrit of large vessel blood was subnormal, and the arterial hematocrit was 43.1.

Dog 25–129 died in shock after a large trans-

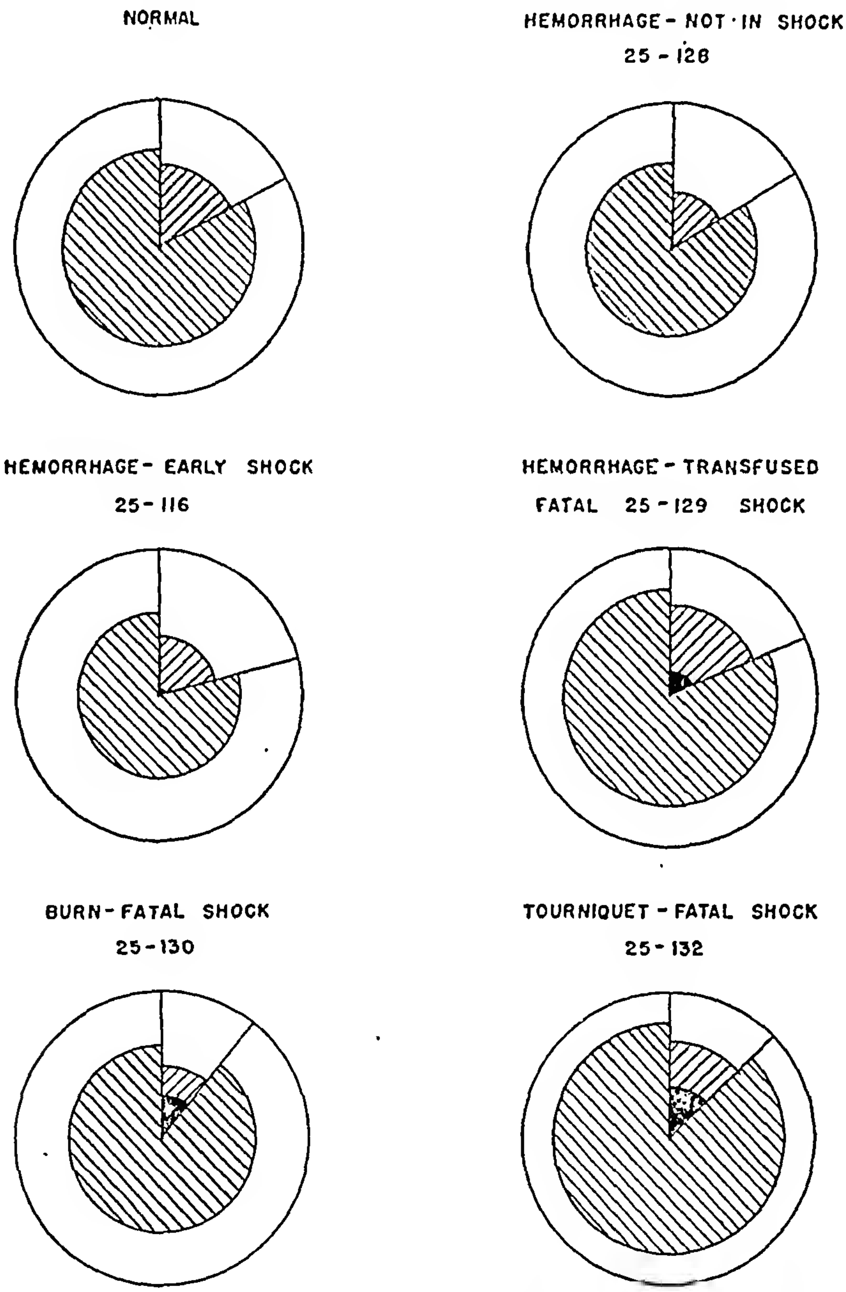

FIG. 1. REDISTRIBUTION OF CELLS AND PLASMA BETWEEN LARGE AND MINUTE VESSELS IN SHOCK

The whole circles represent the total whole blood volume as finally determined by dye plasma, radio-iodo-plasma, and radio-iron red cell volume measurements. The larger segments represent whole blood in all the large vessels; the smaller, whole blood in all the minute vessels. In both segments, the cross-hatched areas represent the red cell fraction of whole blood. The inner black segments represent the fraction of total cells trapped in all the minute vessels. For discussion of changes in the several types of shock, see text.

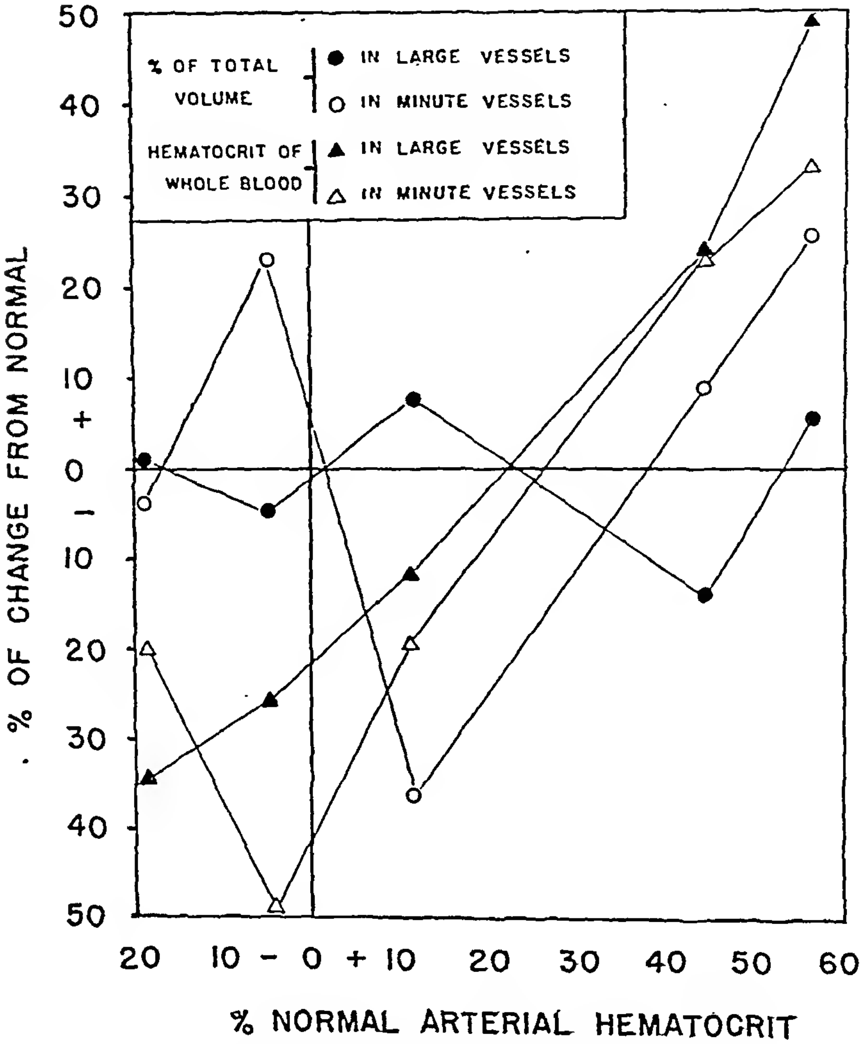

FIG. 2. REDISTRIBUTION OF BLOOD IN LARGE AND MINUTE VESSELS IN RELATION TO ARTERIAL HEMATOCRIT LEVEL IN SHOCK

The change from the normal proportion of whole blood in all the large and minute vessels in shock bears no relation to hemodilution or hemoconcentration. The change from the normal hematocrit of blood in both the large and minute vessels is related to arterial hematocrit level, the hematocrits being low in hemodilution (due here to hemorrhage) and rising progressively with increasing hemoconcentration (here due to plasma loss).

fusion. The percentage of total final blood volume in the minute vessels was well above normal, as was also the hematocrit, and extensive trapping occurred. The hematocrit of large vessel blood was considerably higher than normal. The arterial hematocrit was 62.5.

Dog 25–130 died in shock of severe burns. The minute vessel whole blood percentage was reduced, the hematocrit was normal, but 40 per cent of the cells were trapped. The large vessel hematocrit was normal and the arterial hematocrit was 48.4.

Finally, Dog 25–132 succumbed to tourniquet shock. The minute vessel whole blood percentage was reduced, the hematocrit of this blood was high, and there was considerable trapping. The large vessel hematocrit was extremely high, and the arterial hematocrit was 67.5.

Thus in the 4 animals in recognizable clinical shock *there was not only a reduction in total vol-*

ume, with varying degrees of trapping, but an abnormal distribution of whole blood between large and small vessels, and a resulting distortion of the hematocrit of large and minute vessel blood.

The graphs also emphasize the extremely small fraction of total red cell volume that need be trapped in minute vessels to bring about the reduction in capillary flow associated with irreversible shock.

The degree of abnormal distribution of blood and the distortion in large and minute vessel hematocrit are shown in relation to the level of arterial hematocrit in Figure 2, expressed as percentage deviation from average normal values. Fluctuations in either large or minute vessel whole blood content bore no relationship to arterial hematocrit level. There was, however, a definite direct linear relationship between arterial hematocrit and both large and small vessel hematocrits.

The degree of trapping found in the minute vessels of the organs analyzed did not appear to be related to the amount of external blood loss, as shown in Figure 3. The lowest ratio of circulating to total red cells was found in the burned dog which was bled only 14 per cent of its total volume; the highest, in an animal bled 48 per cent of its volume. The phenomenon is, however, apparently related to the fall in mean arterial pressure, as is also shown in Figure 3. Four dogs with ratios higher than 0.80 had a decline in mean arterial pressure of less than 30 mm. Hg. The remaining 6 dogs, with ratios lower than 0.80, experienced declines of greater than 65 mm. Hg in mean arterial pressure. It has been pointed out (38) that the blood pressure may be a misleading guide to the state of the animal when the blood viscosity is abnormally high due to a high hematocrit. In such event, the blood flow, as indicated by cardiac output, is very poor in spite of nearly normal blood pressure.

Thus, 2 observations appear to be of value in the clinical diagnosis of the presence of, and appraisal of, the severity of capillary trapping in shock: hemoconcentration and arterial hypotension.

It appears probable that while reduction in total volume may be the precipitating cause of peripheral vascular collapse, this simple oligemia alone does not bring about the changes usually classified as "irreversible." It seems far more probable that the reduced flow of whole blood through the nutrient capillaries of all the organs results in anoxia, and starvation of parenchymal cells. If sufficient in degree and duration, impairment of cellular function and eventually death are inevitable sequelae.

If this conception be true, then effective therapy should be directed not only at restoration of re-

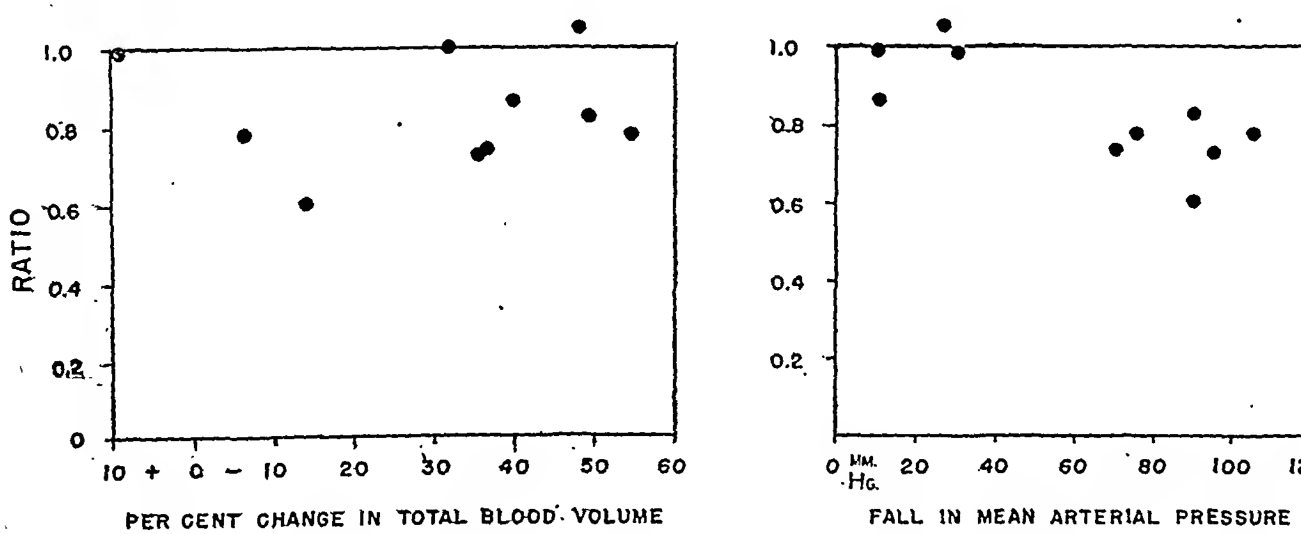

FIG. 3. THE RATIO OF CIRCULATING TO TOTAL RED CELLS IN MINUTE VESSELS IN RELATION TO REDUCTION IN TOTAL BLOOD VOLUME AND FALL IN MEAN ARTERIAL PRESSURE

Reduction in total blood volume alone does not appear to be a causative factor in bringing about trapping of red cells in minute vessels. The degree of trapping does, however, become increasingly severe with progressive fall in mean arterial pressure.

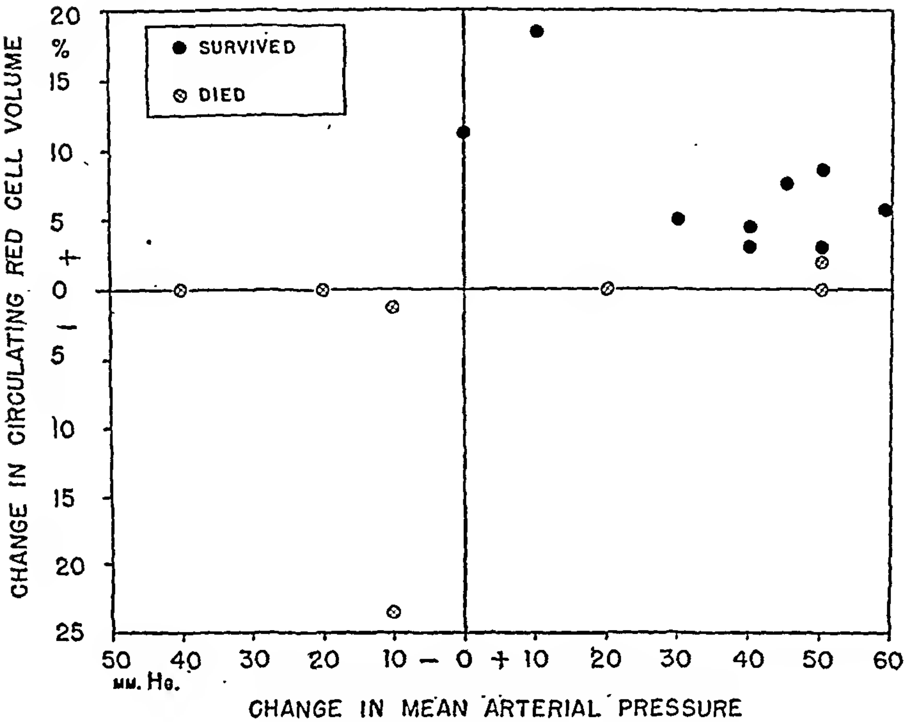

FIG. 4. CHANGE IN TOTAL CIRCULATING RED CELL VOLUME IN RELATION TO CHANGE IN MEAN ARTERIAL PRESSURE IN TOURNIQUET SHOCK AFTER ALBUMIN THERAPY

All of the 8 surviving dogs mobilized red cells, as evidenced by increases in circulating red cell volume, and 7 had sustained rises in mean arterial pressure. Slight increases in red cell volume occurred in only 2 of the 8 dogs that succumbed, although 4 had sustained rises in pressure.

duced total cell and plasma volume, but in restoration of *normal* flow through the entire vascular bed. Following the treatment of shock with protein solutions, there should be an increase, over shock levels, not only in plasma volume but in *circulating red cell volume* if therapy were to be considered successful.

Data obtained in Series IV are of interest. Most of the animals showed increases in mean arterial pressure immediately after the administration of albumin. These pressure rises were fairly well maintained in the survivors but were transient in those that died. There was no relationship between degree of fall in auricular hematocrit and the percentage change from expected circulating red cell volume. The fall in hematocrit in the dogs that died was as great as in the dogs that survived. The improvement in circulating red cell volume was however related to the change in mean arterial pressure. All of the 8 surviving dogs had increases in red cell volume. In 1 the pressure was unchanged but in the other 7, a rise of from 10 to 60 mm. Hg was sustained. Of the 8 dogs that died, 4 had increases in pressure of from 20 to 50 mm. Hg, and 4 had decreases of from 10 to 40 mm. Hg. Three dogs had elevations in pressure of 50 mm. Hg, accompanied by slight increases in red cell volume, but died.

Most of the survivors showed percentage increases in red cell volume that were within the limit of error of the technique of cell volume measurement used. However, the percentage increases are of the same order of magnitude as the percentages of red cells found trapped in minute vessels in Series II and III. The consistent finding of an augmented circulating cell volume after therapy in all the surviving animals is in keeping with the possibility that one effect of successful treatment was to restore the circulation through

the minute vessels to normal, with the resultant flushing back into circulation of trapped cells. Failure of this in the animals that died is of almost equal significance.

In a previous communication (26) it was noted that in the normal dog, the hematocrits of the blood in large vessels and in all the body are approximately equal, but less than the auricular and greater than the minute vessel hematocrits. It was stated above that the ratios of these hematocrits to the auricular hematocrit explained satisfactorily why the dye plasma hematocrit technique always gives a value of the red cell volume some 20 per cent higher than does the radio-iron technique.

In the shocked dogs, the ratios of the large and minute vessel hematocrit to the auricular hematocrit were changed, that of the large vessel hematocrit being 0.83, and that of the minute vessel hematocrit being 0.61.

In the 5 dogs in Series III, the percentage of total circulating whole blood in the minute vessels averaged 16 per cent. Then

$$\begin{aligned} 0.84 \times 0.83 &= 0.697 \\ 0.16 \times 0.61 &= \underline{0.097} \\ 0.794 &= \frac{\text{Vrr}}{\text{Vrpd}} \quad \text{or} \quad \frac{\text{Vrpd}}{\text{Vrr}} = 1.26. \end{aligned}$$

Since here again Vrpd is based on the plasma volume and auricular hematocrit, it follows that Vrr will be about 20 per cent lower than Vrpd. In other words, the red cell volume as measured by the dye technique will be about 26 per cent higher than by the radio-iron technique.

In a series of 28 simultaneous measurements of cell volume by both techniques in dogs in varying degrees of shock, in which auricular hemaocrits ranged from 19 to 69, averaging 43.7, the average ratio of $\frac{\text{Vrpd}}{\text{Vrr}}$ was found to be 1.32.

Thus, the former technique not only over-estimates circulating red cell volume in the normal state, but gives an even higher degree of error in peripheral vascular collapse, regardless of the presence of hemodilution or hemoconcentration.

COMMENT

These studies are in keeping with the findings of several authors on the circulatory changes in shock. The gross volume studies confirm the conclusions of Noble and Gregersen (22) that reduction in blood volume is due to loss of both cells and plasma: whole blood. In these controlled experiments the loss of whole blood was always more than the measured external blood loss. In certain types of insult (burns, tourniquet and toxins), some of this whole blood was undoubtedly lost in damaged areas or in the intestinal tract. The other site of whole blood "loss" lies within the capillary bed. Chambers *et al* (39) found cessation of blood flow in the capillaries in the mesentery of dogs in shock. The studies herein reported indicate that this phenomenon is not confined to sites of injury, or to abdominal viscera, but that it is a widespread phenomenon occurring in all organs throughout the body.

Since the methods of volume measurement used are dynamic, they reveal changes in total blood flow through capillary beds of all the organs. The net effect of trapping is a reduction of flow through vital areas. The resulting anoxia, failure in nutrition and waste disposal, if continued for long periods, is not compatible with the continued existence of certain parenchymal cells. For instance, it has been shown (37) that liver cells in dogs in hemorrhagic shock may be the earliest to show the effects of the reduced blood flow characteristic of peripheral vascular collapse.

Since the capillaries of organs normally contain less than $\frac{1}{5}$ of all the total circulating cells and plasma, the trapping of only $\frac{1}{20}$ of the total blood volume may bring about a reduction in flow to $\frac{1}{4}$ or $\frac{1}{3}$ of normal. Extensive trapping may be present in the absence of measurable hemoconcentration or hemodilution, or with a normal hematocrit.

Effective therapy should be directed not only towards restoration of total blood volume, but to the resumption of normal capillary flow. Objective evidence of effective therapy lies in the restoration and maintenance of normal hematocrit and normal arterial pressure.

CONCLUSIONS

1. Measurements of the total circulating red cell and plasma volume, and of the distribution of blood in large and minute vessels were made in dogs in experimental shock, by means of tech-

niques employing Evans blue, 2 isotopes of radioactive iron, and radioactive iodine. The following characteristics of the circulation in shock have been demonstrated.

2. There is always a greater reduction in circulating red cells and plasma than can be accounted for by measured external blood loss.

3. A part of this discrepancy in blood volume may be lost into damaged areas or by intestinal bleeding in certain types of shock.

4. Regardless of etiology, there is a widespread trapping of red cells within the minute vessels of all the organs in the body.

5. The effect of this trapping is a reduction in capillary blood flow through all organs.

6. Since the normal capillary blood content is less than 20 per cent of the total volume, the trapping of even a small portion thereof may result in a fatal reduction in capillary flow.

7. There is an abnormal distribution of whole blood between large and minute vessels which is not reflected by changes in the arterial hematocrit.

8. There are marked deviations from normal in the hematocrit of large and small vessel blood, and these are directly related to changes in the arterial hematocrit.

9. The degree of red cell trapping present does not parallel the reduction in total blood volume, but is related to the reduction in mean arterial pressure. However, this correlation between trapping and arterial pressure may not apply when the hematocrit is abnormally high.

10. The findings indicate that the therapy of shock should be directed not only towards restoration of total blood volume, but also towards the resumption and maintenance of normal capillary flow.

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BIBLIOGRAPHY

1. Keith, N. M., Rowntree, L. G., and Geraghty, J. T., A method for the determination of plasma and blood volume. Arch. Int. Med., 1915, 16, 547.
2. Robertson, O. H., and Bock, A. V., Blood volume in wounded soldiers. I. Blood volume and related blood changes after hemorrhage. J. Exper. Med., 1919, 29, 139.
3. Robertson, O. H., and Bock, A. V., Memorandum on blood volume. Med. Research Committee Special Report, No. 25, 1918.
4. Gregersen, M. I., Traumatic shock. Bull. New York Acad. Med., 1943, 19, 666.
5. Cannon, W. B., Traumatic Shock. D. Appleton and Company, New York, 1923.
6. Blalock, A., Experimental shock. The cause of low blood pressure produced by muscle injury. Arch. Surg., 1930, 20, 959.
7. Blalock, A., Trauma to the intestines. The importance of the local loss of fluid in the production of low blood pressure. Arch. Surg., 1931, 22, 314.
8. Moon, V. H., Dynamics of Shock and its Clinical Implications. Lea and Febiger, Philadelphia, 1942.
9. Gibson, J. G., 2nd, and Evans, W. A., Jr., Clinical studies of the blood volume. I. Clinical application of a method employing the azo dye "Evans blue" and the spectrophotometer. J. Clin. Invest., 1937, 16, 301.
10. Gregersen, M. I., and Stewart, J. D., Simultaneous determination of the plasma volume with T-1824, and the "available fluid" volume with sodium thiocyanate. Am. J. Physiol., 1939, 125, 142.
11. Davis, L. J., Determination of blood volume in man with "Evans" blue ("T-1824"). Edinburgh M. J., 1942, 49, 465.
12. Gibson, J. G., 2nd, and Branch, C. D., Blood volume changes during surgical procedures. Surg., Gynec. and Obst., 1937, 65, 741.
13. Gibson, J. G., 2nd, and Kopp, I., Studies in the physiology of artificial fever. I. Changes in the blood volume and water balance. J. Clin. Invest., 1938, 17, 219.
14. Keeley, J. L., Gibson, J. G., 2nd, and Pijoan, M., The effect of thermal trauma on blood volume, serum protein, and certain blood electrolytes: An experimental study of the effect of burns. Surgery, 1939, 5, 872.
15. Dunphy, J. E., and Gibson, J. G., 2nd, The effect of replacement therapy in experimental shock. Surgery, 1941, 10, 108.
16. Scudder, J., Shock: Blood Studies as a Guide to Therapy. J. B. Lippincott Company, Philadelphia, 1940.
17. Harkins, H. N., Recent advances in the study and management of traumatic shock. Surgery, 1941, 9, 231.
18. Wiggers, C. J., The present status of the shock problem. Physiol. Rev., 1942, 22, 74.
19. Mahoney, E. B., Kingsley, H. D., and Howland, J. W., The treatment of experimental shock by the intravenous injection of dilute, normal, and concentrated plasma. Surg., Gynec. and Obst., 1942, 74, 319.
20. Richards, D. W., Jr., The circulation in traumatic shock in man. The Harvey Lectures, 1943-1944, p. 217. The Science Press Printing Company, Lancaster, Pa.

21. Evans, E. I., Hoover, M. J., James, G. W., and Alm, T., Studies on traumatic shock. I. Blood volume changes in traumatic shock. Ann. Surg., 1944, 119, 64.
22. Noble, R. P., and Gregersen, M. I., with the technical assistance of Porter, P. M., and Buckman, A., Blood volume in clinical shock. II. The extent and cause of blood volume reduction in traumatic, hemorrhagic, and burn shock. J. Clin. Invest., 1946, 25, 172.
23. Fine, J., and Seligman, A. M., Traumatic shock: IV. A study of the problem of the "lost plasma" in hemorrhagic shock by the use of radioactive plasma protein. J. Clin. Invest., 1943, 22, 285.
24. Fine, J., and Seligman, A. M., Traumatic shock. An experimental study including evidence against the capillary leakage hypothesis. Ann. Surg., 1943, 118, 238.
25. Fine, J., and Seligman, A. M., Traumatic shock. VII. A study of the problem of the "lost plasma" in hemorrhagic, tourniquet, and burn shock by the use of radioactive iodo-plasma protein. J. Clin. Invest., 1944, 23, 720.
26. Gibson, J. G., 2nd, Peacock, W. C., Seligman, A. M., and Sack, T., Circulating red cell volume measured simultaneously by the radioactive iron and dye methods. J. Clin. Invest., 1946, 25, 838.
27. Root, G. T., and Mann, F. C., An experimental study of shock with special reference to its effect on the capillary bed. Surgery, 1942, 12, 861.
28. Chambers, R., Zweifach, B. W., and Lowenstein, B. E., The peripheral circulation during the tourniquet shock syndrome in the rat. Ann. Surg., 1944, 20, 791.
29. Gibson, J. G., 2nd, Seligman, A. M., Peacock, W. C., Aub, J. C., Fine, J., and Evans, R. D., The distribution of red cells and plasma in large and minute vessels of the normal dog, determined by radioactive isotopes of iron and iodine. J. Clin. Invest., 1946, 25, 848.
30. Gibson, J. G., 2nd, and Evelyn, K. A., Clinical studies of the blood volume. IV. Adaptation of the method to the photoelectric microcolorimeter. J. Clin. Invest., 1938, 17, 153.
31. Gibson, J. G., 2nd, Weiss, S., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., Good, W. M., and Kip, A. F., The measurement of the circulating red cell volume by means of two radioactive istopes of iron. J. Clin. Invest., 1946, 25, 616.
32. Duncan, G. W., and Blalock, A., The uniform production of experimental shock by crush injury: possible relationship to clinical crush syndrome. Ann. Surg., 1942, 115, 684.
33. Nathanson, I. T., Nutt, A. L., Pope, A., Zamecnik, P. C., Aub, J. C., Brues, A. M., and Kety, S. S., The toxic factors in experimental traumatic shock. I. Physiologic effects of muscle ligation in the dog. J. Clin. Invest., 1945, 24, 829.
34. Fine, J., Frank, H. A., and Seligman, A. M., Traumatic shock. VIII. Studies in the therapy and hemodynamics of tourniquet shock. J. Clin. Invest., 1944, 23, 731.
35. Freedberg, A. S., Haimovici, H. (by invitation), and Blumgart, H. L., The cardiovascular dynamics in shock due to infection. J. Clin. Invest., 1944, 23, 930.
36. Aub, J. C., Brues, A. M., Kety, S. S., Nathanson, I. T., Nutt, A. L., Pope, A., and Zamecnik, P. C., The toxic factors in experimental traumatic shock. IV. The effects of the intravenous injection of the effusion from ischemic muscle. J. Clin. Invest., 1945, 24, 845.
37. Frank, H. A., Seligman, A. M., and Fine, J., Traumatic shock. XIII. The prevention of irreversibility in hemorrhagic shock by vivi-perfusion of the liver. J. Clin. Invest., 1946, 25, 22.
38. Seligman, A. M., Frank, H. A., and Fine, J., Traumatic shock. XII. Hemodynamic effects of alterations of blood viscosity in normal dogs and in dogs in shock. J. Clin. Invest., 1946, 25, 1.
39. Chambers, R., Zweifach, B. W., and Lowenstein, B. E., Circulatory reactions of rats traumatized in the Noble-Collip drum. Am. J. Physiol., 1943, 139, 123.

A STUDY OF THE RESIDUAL EFFECTS OF PHOSGENE POISONING IN HUMAN SUBJECTS. I. AFTER ACUTE EXPOSURE

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INTRODUCTION

During World War I the British Chemical Warfare Medical Committee (1 to 8) recognized a distinctive syndrome which resulted most often from exposure to phosgene and which resembled in symptomatology the "Irritable Heart" or "Effort Syndrome." The patients presented scant physical evidence of structural disease of the lungs. Haldane and his co-workers emphasized that these symptoms were associated with rapid, shallow breathing (3). Although no measurements of the oxygen of the blood were reported, the cyanosis, polycythemia, and relief after inhalation of oxygen suggested that anoxemia was an important factor (2, 6). Furthermore, it was suggested that rapid, shallow breathing alone might produce anoxemia (5). Haldane, Meakins, and Priestley (5) state that "as soon as the exaggerated Hering-Breuer reflex has been produced by gassing or any other cause, the resulting slight anoxemia produces a general nervous upset in which various reflexes tend to be abnormal or exaggerated. The exaggeration of the Hering-Breuer reflex itself may thus be kept up and with it the anoxemia." The patients were treated with oxygen inhalation. They were encouraged to take gradually increasing exercise. The majority of patients improved, but a considerable number were unable to return to duty (1 to 8).

The British Ministry of Pensions maintained that serious structural after-effects did not occur, provided the lungs were healthy before gassing. Only a few casualties suffered from chronic bronchitic attacks (9, 10). On the other hand, numerous reports in the French and American literature have presented clinical evidence of residual damage to the lungs among phosgene and other types of chemical warfare casualties in the form of one or more of the following: chronic bronchitis, pulmonary emphysema, obliterative bronchiolitis, peribronchiolitis, bronchiectasis and suppuration of the lungs (11 to 18).

In spite of these valuable observations, there are too few objective measurements to indicate whether such patients have structural damage to the lungs, or simply a reflex or psychological disturbance. With the development of more precise methods for the evaluation of the ventilation of the lungs and the aeration of the blood, it is possible to study in greater detail these aspects in patients after exposure to phosgene. The cardiovascular system and the patients' psychological reactions also warrant more detailed study.

EXPERIMENTAL

Six patients, who are known to have been exposed to phosgene and who developed symptoms of acute poisoning, volunteered for the studies here reported.

METHODS

1. PULMONARY FUNCTION TESTS. These consisted of ventilatory function tests which are concerned with movement of air into and out of the lungs and respiratory function tests which are concerned with exchange of oxygen and carbon dioxide in the lungs.

A. Ventilatory function tests.

a. Lung volume measurements. Vital capacity, complementary air, tidal air, and reserve air were measured from graphic tracings of a spirometer. All volumes are expressed as gas saturated with water vapor at 37° C. and prevailing barometric pressure.

b. Mid-capacity (functional residual air). This was measured by the method of Darling, Cournand, and

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Richards (19). The mid-capacity minus the reserve air represents residual air.[3]

c. Respiratory dead space. This was calculated according to Bohr's formula (23). It included an instrumental dead space of 100 ml.

d. Intrapulmonary mixing of gases. This was studied by the method of Darling, Cournand, and Richards (24). The presentation of the mixing curves has been changed by reversing the axes of the semi-logarithmic scale. This changes the sigmoid curve used by Darling and his coworkers (24) to a much simpler form consisting chiefly of two straight lines, the falling line of nitrogen dilution and the final level reached when the only remaining nitrogen is that eliminated from the blood. This curve is easier to construct, only two points being needed to define its form. It has the additional advantage that all deviations from the predicted values are represented in proportion to their significance, regardless of the level of alveolar nitrogen.

e. Completeness of emptying of lung nitrogen. This was measured as described by Cournand *et al.* (20).

f. Rate and depth of respiration. These were measured in a recording spirometer at rest, during exercise, and on recovery from exercise.

g. Voluntary breathing capacity (maximum breathing capacity). This was measured according to Cournand and Richards (25). These investigators kindly sent us the formulae for prediction of maximum breathing capacity.

h. Breath-holding. This was performed in moderate inspiration. At the end, a sample of alveolar air was collected and analyzed for oxygen and carbon dioxide content. Observations were also made on the effect of hyperventilation, for one minute, and oxygen breathing for two or three minutes, singly or combined on the duration of breath-holding. A breath-holding time of less than 25–30 seconds when breathing room air was considered limited.

B. Respiratory function tests.

a. Oxygen content and capacity of arterial blood. These were each measured in 1 ml. of blood (26).

b. Tension of oxygen and carbon dioxide in arterial blood. These were measured according to the method of Riley (27). In most instances duplicate samples of blood were drawn a few minutes apart. The post exercise arterial blood samples were usually obtained at ½ to 1 minute and 3 to 5 minutes after exercise. Alveolar air was collected at the end of withdrawal of blood. At rest, the air was collected at the end of expiration; after exercise, at the height of inspiration. The range of oxygen tension values of arterial blood for each observation listed in the tables is in most instances based on three analyses, occasionally on two, four or five.[4]

c. pH, carbon dioxide content, and tension of plasma. Arterial blood was drawn anaerobically into a syringe wet with heparin. The pH determination was made at room temperature with a sealed glass electrode which was standardized empirically with plasma equilibrated at 37° C. with gas of known partial pressure of carbon dioxide. The carbon dioxide content of the plasma was measured (26) and its carbon dioxide tension was calculated from Peters' chart (31).

d. The oxygen extraction and carbon dioxide output per volume of air breathed at rest, during exercise and recovery. These were measured on expired air collected in a spirometer. At various times, a bicycle ergometer, a flight of steps, and single step (25) were used depending upon the degree of exercise desired. In the oxygen tension studies, exercise was continued for 30 seconds beyond the time the patient experienced marked shortness of breath. The rate, depth, and minute volume of respiration was recorded at the start of the exercise and during the last 30 seconds. Expired air was collected until the alveolar air sample was taken. The range of normal values for the step test (25) based on unpublished data was kindly sent to us by Drs. Cournand and Richards.

2. CIRCULATORY FUNCTION STUDIES.

a. Postural tests. The patient lay on a tilt table while observations of pulse and arterial blood pressure and recordings of rate and depth of breathing were made. When a steady base line had been established the patient was shifted from the horizontal to the 90° vertical position. When the circulation and breathing reached a constant level, the patient was returned to the horizontal position where similar observations were made.

b. Ballistocardiographic studies of cardiac output. These were performed by Dr. Giles F. Filley on an instrument designed by Dr. Dugald S. Brown (32) and calibrated to correspond with Starr's instrument. Calculations were based on the area formula (33).

[3] The published data on the per cent of the total lung volume comprised of residual air in normal individuals show a wide range of variation (20, 21, 22). Moreover there has been some variation in results with the same method in different laboratories (21, 22) (Figure 1). This may be due to the fact that the number of individuals studied in each age group has not been comparable. Furthermore, differences in method may lead to some divergence of results, particularly in instances of pulmonary disease (20). There are enough data with each method to reach a general idea of the mean and range, but not enough to be completely satisfactory. It is necessary, therefore, to withhold final judgment on borderline abnormalities until more data can be obtained on normal individuals of comparable age with the same technique as used on the patients in this study (Figure 1).

[4] In 21 comparisons performed in 17 healthy resting subjects, the alveolar air pO_2 range was 89 to 103 mm. Hg, average 99 mm. Hg and the arterial blood pO_2 range was 87 to 101 mm. Hg, average 98 mm. Hg. These agree essentially with similar studies recently reported (28, 29). In 11 comparisons in 9 healthy subjects, post exercise, the alveolar air pO_2 range was 98 to 118 mm. Hg, average 115 mm. Hg and the arterial blood pO_2 range was 92 to 114 mm. Hg, average 110 mm. Hg. The post-exercise studies were carried out in subjects who had volunteered for the studies at rest (30).

3. PSYCHIATRIC EXAMINATIONS.

These were performed in all 6 patients and in patients 2, 3, 4, 5, of the chronically exposed group (34) by Dr. Nicholas L. Ballich. The psychiatric summaries are presented in the Appendix.

4. OTHER STUDIES.

In all patients roentgenograms of the chest and electrocardiograms were taken. Blood counts, blood chemical studies, urine examinations, and other studies were performed as indicated.

RESULTS

The results are presented in detail in the Appendix and are summarized in Table I.

TABLE I

Summary of clinical observations and data on studies performed

Case Number*	1	2	3	4	5	6
Patient's initials	E.T.	L.W.	H.G.	L.T.	S.O.	M.P.
Age	38	39	30	48	43	49
Months after accident**	14	6	6	3	5	5
Months worked with phosgene	6	12	18	24	2	1
Chronic symptoms	A	N	N	A	A	A
Physical signs: Acute	A	A	A	A	N	N
Physical signs: Chronic	N	N	N	B	N	N
Roentgenogram of chest	N	N	N	N	N	N
Volume: vital capacity	N	N	N	A	N	N
Per cent residual air / Total capacity	B	N	N	B	N	N
Intrapulmonary mixing of gases	N	N	B	A	N	B
Pulmonary emptying	N	N	N	A	N	B
Resting pattern of breathing: High rate	N	A	A	A	A	A
Resting pattern of breathing: Low tidal air	N	N	A	B	A	A
Resting pattern of breathing: High min. volume	N	A	A	A	A	A
Resting pattern of breathing: Low O_2 extraction	B	A	A	B	A	A
Exercise pattern of breathing: High rate	N	B	B	B	A	A
Exercise pattern of breathing: Low tidal air	B	N	B	B	N	A
Exercise pattern of breathing: Low O_2 extraction	N	N	N	B	A	A
Arterial blood: Rest	N	A	N	N	A	N
Arterial blood: Exercise	N	A	N	N	N	—
Arterial blood: Oxygen	—	N	—	N	A	—
Breath-holding	N	N	N	A	A	N
Voluntary breathing capacity	N	A	A	N	A	N
Postural tests	N	N	N	N	N	N
Cardiac output	N	A	N	N	—	N

A = Definitely abnormal. B = Borderline abnormal.
N = Normal. — = Not done.

* Listed in order of severity of exposure.
** Applies to all special studies except arterial blood and alveolar air oxygen and carbon dioxide tensions and cardiac output which were performed 4 to 8 months later. Symptoms, physical and roentgenographic findings were unchanged on re-examination of all available patients (All except 5) 4 to 8 months later.

DISCUSSION

This small group of patients presents symptoms very similar to those observed after phosgene poisoning in World War I (1 to 8). These symptoms are similar from patient to patient, but some individuals appear to suffer much more than others. The patients complained regularly of shortness of breath, tightness of the chest, and precordial pain at rest or after slight exertion. Cough was disturbing, particularly on exertion and at night, but only a little mucoid sputum was produced. Sleep was often disturbed by anxiety, terrifying dreams, dyspnea, and cough. Nausea, vomiting, and diarrhea were occasionally present. Several patients complained of nervousness and fatigue.

The most impressive feature of the physical examination was the rapid, generally shallow breathing, previously described by Haldane *et al.* (5). This pattern of respiration was present during sleep. Breathing was regular and not interrupted by sighs, which have been observed in respiratory neuroses (35, 36, 37). The patients with complaints looked anxious. The hands were often cold and clammy. Otherwise, the physical examination presented no significant abnormality.

There was no tendency to polycythemia such as was observed during World War I (2). The red blood cell count, hemoglobin, and hematocrit were within the normal range or slightly below normal. Arterial and venous pressures were normal. Roentgenograms of the chest did not exhibit any abnormality of the lungs or heart, except for a healed tuberculous focus in two patients.

The studies of pulmonary function revealed a number of borderline changes and frank abnormalities in lung volume, intrapulmonary mixing of gases, and transfer of oxygen to the arterial blood. Some abnormality was observed in every patient, but there was no consistent pattern and the severity of the changes could not be correlated with the patients' symptoms.

The most striking and consistent abnormalities brought out by these studies were concerned with the rapid and usually shallow pattern of respiration. This pattern was present both at rest and during exercise and was accompanied by a low extraction of oxygen and a low output of carbon dioxide per unit of air breathed. These abnormalities are closely related and have been described after phosgene poisoning (3, 5). In contrast to Haldane's findings (3), however, the unusual pattern of respiration was not altered by breathing pure oxygen, and could be voluntarily slowed and deepened to a normal level. The limitation of breath-holding to a few seconds, described by Haldane *et al.* (5), was not observed in our patients, who could generally hold a breath for a

normal time and who responded to hyperventilation or breathing oxygen with a prolongation of breath-holding time. Voluntary breathing capacity was significantly reduced in three of the six patients (cases 2, 3, 5). It is of interest that these three patients had a normal vital capacity.

The alveolar air oxygen tension was normal or high in this group of patients. Haldane and his co-workers (3) made a similar observation and remarked upon the paradox of a normal alveolar air and apparent anoxemia without obvious anatomical disease of the lungs. They postulated unequal ventilation during shallow breathing in which certain alveoli were poorly ventilated and contributed little to the alveolar air specimen, which came primarily from well-ventilated alveoli. This inefficient pulmonary ventilation resulted in anoxemia. Certain indirect evidence was brought forward to support this view (38). Some observations on our patients do not substantiate certain of Haldane's postulates.

Anoxemia was present in only one of the six patients (case 5). In this patient, intrapulmonary mixing of gases was normal as measured by the nitrogen dilution method. In one other patient (case 2) who did not exhibit anoxemia, a disturbance in the transfer of oxygen to arterial blood existed without demonstrably impaired intrapulmonary mixing of gases. It is therefore apparent that in our group of patients, anoxemia is infrequent and is not necessarily dependent on impaired mixing of gases in the lung. A well-marked defect of intrapulmonary mixing of gases was not associated with anoxemia, even with shallow breathing, in three patients chronically exposed to phosgene (34).

Electroencephalograms and conditioned reflex studies, designed to detect possible diffuse cortical damage resulting from anoxemia, showed minimal changes in the acute stage and essentially normal patterns when repeated several weeks later. On the basis of these tests, it was not possible to demonstrate any clear-cut, irreversible, organic involvement of the cerebral cortex.

Although the nitrogen-dilution method does not show a consistent abnormality of intrapulmonary mixing of gases during shallow breathing, a tendency toward impaired mixing during shallow breathing is apparent when all observations on patients exposed to phosgene are charted (Figure 2).

Exercise to the point of dyspnea, then maintained for a half minute, did not produce anoxemia in any patient and actually abolished the anoxemia in one patient (case 5).

Postural tests indicated that there was no instability of vascular control nor any accompanying respiratory change. The ballistocardiographic tracings indicated a subnormal cardiac output in one patient (case 2). No case showed the changes described by Starr in a large proportion of his cases of neurocirculatory asthenia (39).

The results of these studies demonstrate certain disturbances of pulmonary function which were not recognized on clinical examination. The changes were probably not serious enough to account for the disabling symptoms present in some of the patients. There was no obvious change in symptoms, physical findings, or roentgenograms for as long as a year and a half after exposure, but a detailed reexamination of the patients after a number of years would be necessary to decide whether the disease would recede or progress.

The correlation of the psychiatric abnormalities with the severity of symptoms was quite obvious. The timid and apprehensive patients developed disabling hypochondriacal and anxiety reactions. Pre-existing maladjustments and insecurity helped to perpetuate the symptoms. Socio-economic factors and previous personality trends were outstandingly important. Visible emotional support in the form of substantial personal contacts tended to stabilize the patient's reactions. Hospitalization and sympathy resulted in minimal improvement of symptoms and an increasing tendency toward chronic invalidism. Although compensation was rarely mentioned, the potential financial and psychological gains of invalidism loomed large to the insecure patients.

Three important points may be drawn from these data. Definite abnormalities in pulmonary function may persist for at least a year after exposure to phosgene. A characteristic change in the pattern of breathing accompanies these changes in the lungs. Symptoms are quite uniform in type, but the severity of symptoms and the ensuing disability are much more closely related to the patient's psychological reactions than to any demonstrable physical or physiological abnormality.

The disturbances in pulmonary function are almost certainly a reflection of organic changes in the lungs as a result of exposure to phosgene. The disturbances are generally suggestive of damage to the bronchioles and surrounding parenchyma as seen in beginning pulmonary emphysema.

The rapid, shallow breathing is probably also a consequence of pulmonary injury. This is perhaps due to a disturbance in the nerve-endings concerned with the stretch reflex of the lungs (Hering-Breuer). The same pattern of breathing occurs in men chronically exposed to phosgene (34). The persistence of this pattern of breathing during sleep and its occurrence in patients without complaints point strongly to a physiologic rather than a neurotic basis. Furthermore, the graphic respiratory records of our patients after recovery from phosgene poisoning show none of the features of a respiratory neurosis (35, 36, 37). The results of our studies also indicate that anoxemia is not necessarily a consequence of shallow breathing and is not an essential factor for its persistence as suggested by Haldane and his co-workers (5).

Disability in the group of patients is apparently caused by symptoms rather than by any serious inability of the lungs or cardiovascular system to perform adequately. There is a large neurotic component in the symptoms of the disabled patients. Mild symptoms referable to the lungs occur, however, even in stable individuals who have returned to full activity after exposure to phosgene. Even in the absence of serious injury to the lungs, the conscious or unconscious awareness of a disturbance in the pattern of breathing may be responsible for these complaints. In the patients with a suitable psychological predisposition, many of the neurotic complaints are focused on the pulmonary symptoms, which are magnified and extended to the point of complete disability, since the patients are unwilling to attempt any activity for fear of precipitating the very disturbing and even terrifying symptoms. The symptoms in such patients may be partly those of an anxiety neurosis, but still resemble closely the symptoms produced by exposure to phosgene.

Sudden grave exposure to phosgene obviously resulted in great psychic trauma and was very demoralizing. It led to a considerable degree of personality disorganization and to the exaggeration of latent neurotic trends, brought out by this personal stress, which also highlighted socio-economic factors. As the acute anxiety subsided with understanding interviewing and management, there was a shift of emphasis from concern about the physiological manifestations of anxiety to personal issues and themes in the lives of these patients. It may be said that this transition, when accomplished, was of considerable therapeutic value.

CONCLUSIONS

1. Objective studies of pulmonary function have been carried out in six patients from 3 to 14 months after exposure to phosgene, which in 4 patients resulted in acute pulmonary edema and emphysema.

2. After 3 weeks following exposure, physical examinations and roentgenograms revealed no definite evidence of disease of the lungs, but the patients complained of symptoms like those reported in phosgene casualities during World War I.

3. The rapid, shallow breathing, described by Haldane, Meakins, and Priestley, was regularly observed but was not associated with a disturbance of intrapulmonary mixing of gases sufficient to lead to anoxemia.

4. Breathing oxygen did not alter the pattern of respiration and did not afford consistent relief of symptoms.

5. Objective changes of pulmonary function were consistently observed but varied in type and severity, and could not be correlated with the seriousness of initial injury or chronic symptoms.

6. The severity of chronic symptoms and the associated disability were closely related to the patients' psychological reactions.

7. A possible explanation of these observations would be that the injury initiated changes in the lungs and a uniform pattern of symptoms, which in certain individuals were sufficiently disturbing to produce disability.

APPENDIX

Clinical summaries and data on pulmonary and circulatory function studies. Case histories of patients 1 through 6 are given below. The clinical course and laboratory findings of cases 1, 2,

3, 4 and 5 are summarized in Figures 1A through 5A.

The results of the pulmonary function studies on each patient are presented in Figures 1B, 1C, 1D through 5C, 6A, 6B and in Tables IA, IB through IVB, VA, VB, VC through VIC.

The number of each figure and table corresponds to the case number listed in Table I.

Legends for figures on intrapulmonary mixing of gases. The data on each patient are presented in two ways (see Methods).

(a) Figures 1C, 2C, 3C, 4C, 5B, 6A. These figures present the difference between predicted nitrogen concentrations and observed nitrogen concentrations, and between predicted nitrogen concentrations and calculated nitrogen concentrations for each number of breaths. The ordinates represent differences in nitrogen concentration

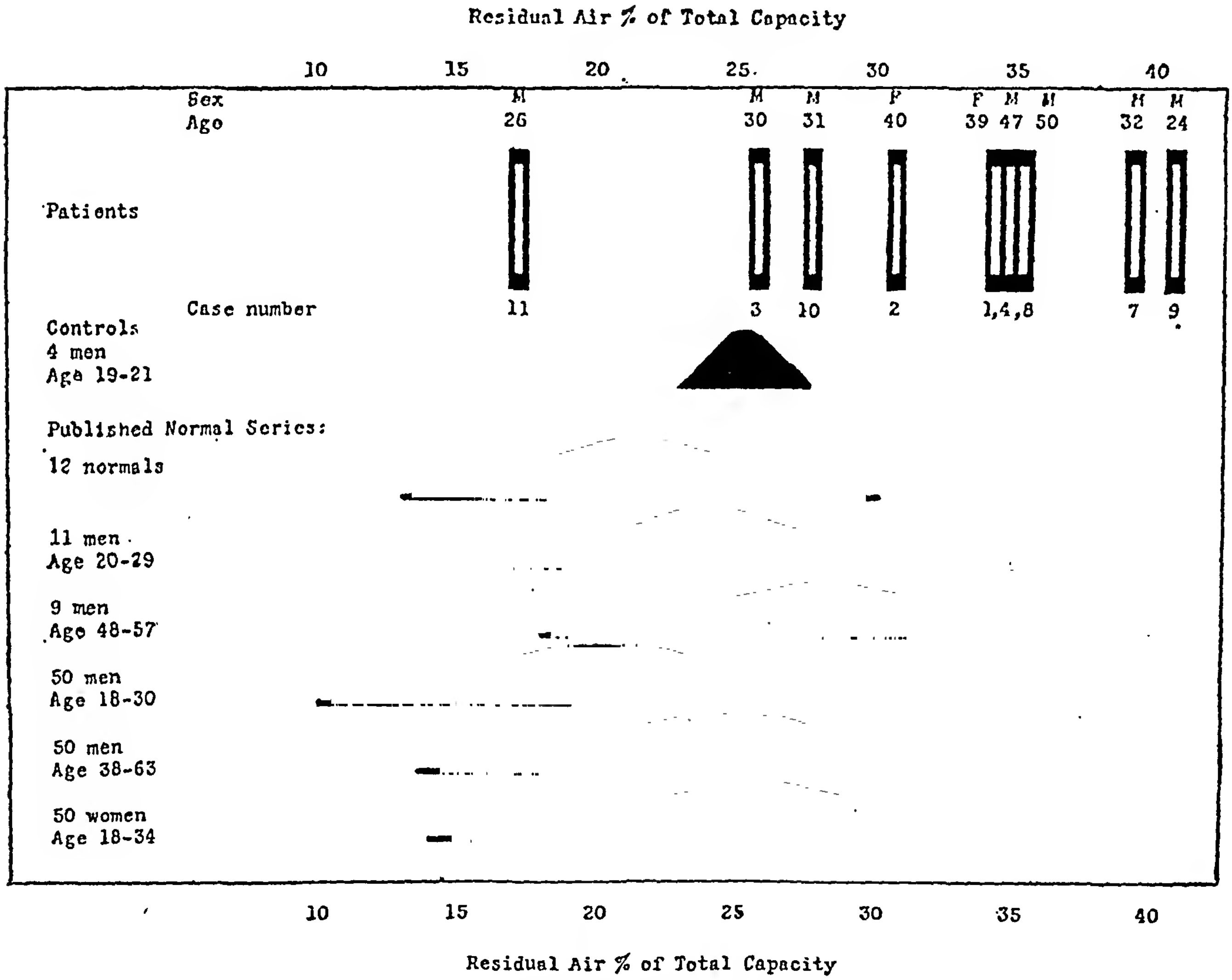


FIG. 1. RESIDUAL AIR AS PER CENT OF TOTAL LUNG CAPACITY IN PATIENTS EXPOSED TO PHOSGENE AND IN NORMAL MEN AND WOMEN

Each block at the top of the chart represents a patient exposed to phosgene. The number under each block designates the patient as referred to in the case history, with the exception that numbers 7, 8, 9, 10, 11 refer to cases 1, 2, 3, 4, 5 in the group of patients with chronic exposure to phosgene (34).

In descending order are curves which represent the mean and range of variation observed in different laboratories: 4 men, 19–21 years, present study, open circuit method of Darling *et al* (19); 12 normals, age not specified, open circuit method of Darling *et al,* by Cournand *et al* (20); 11 men (20–29 years) and 9 men (48–57 years), modified Christie method (closed circuit), by Robinson (23); last three curves, modified Christie method (closed circuit), by Kaltreider *et al* (21).

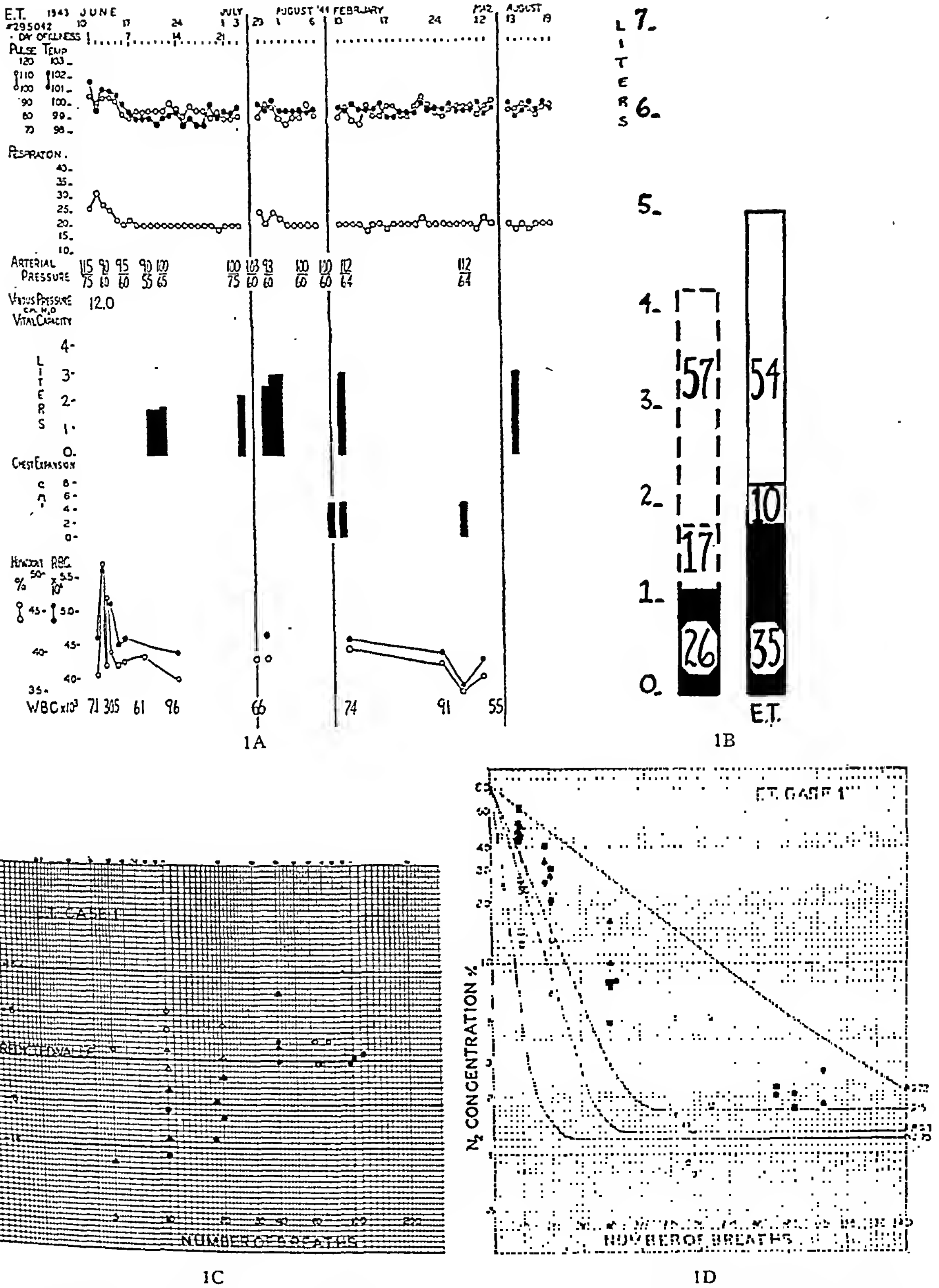


FIG. 1A. CLINICAL COURSE; 1B. SUBDIVISIONS OF LUNG VOLUME; 1C AND 1D. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 220–440 ml. generally 325 ml.) voluntarily deepened: 480–810 ml. (generally 600 ml.). DEAD SPACE: 182 ml. (Tidal Air below 400 ml.), 224 ml. (Tidal Air above 400 ml.).

from the predicted value which is represented by the line passing through the zero point. The abscissae represent the number of breaths.

Solid circle ● = observed alveolar nitrogen − predicted nitrogen concentration.

Solid triangle ▲ = calculated nitrogen − predicted nitrogen concentration. Open circle ○ and open triangle △ represent similar nitrogen concentration differences during voluntary deep breathing.[5]

[5] Comparisons between predicted, observed, and calculated levels of nitrogen remaining in the lungs after a measured number of breaths of 100 per cent oxygen may be made. Predicted nitrogen concentration is the level which would exist in the alveolar air in the presence of theoretically perfect intrapulmonary mixing of gases. This is based on the formula derived by Darling *et al.*

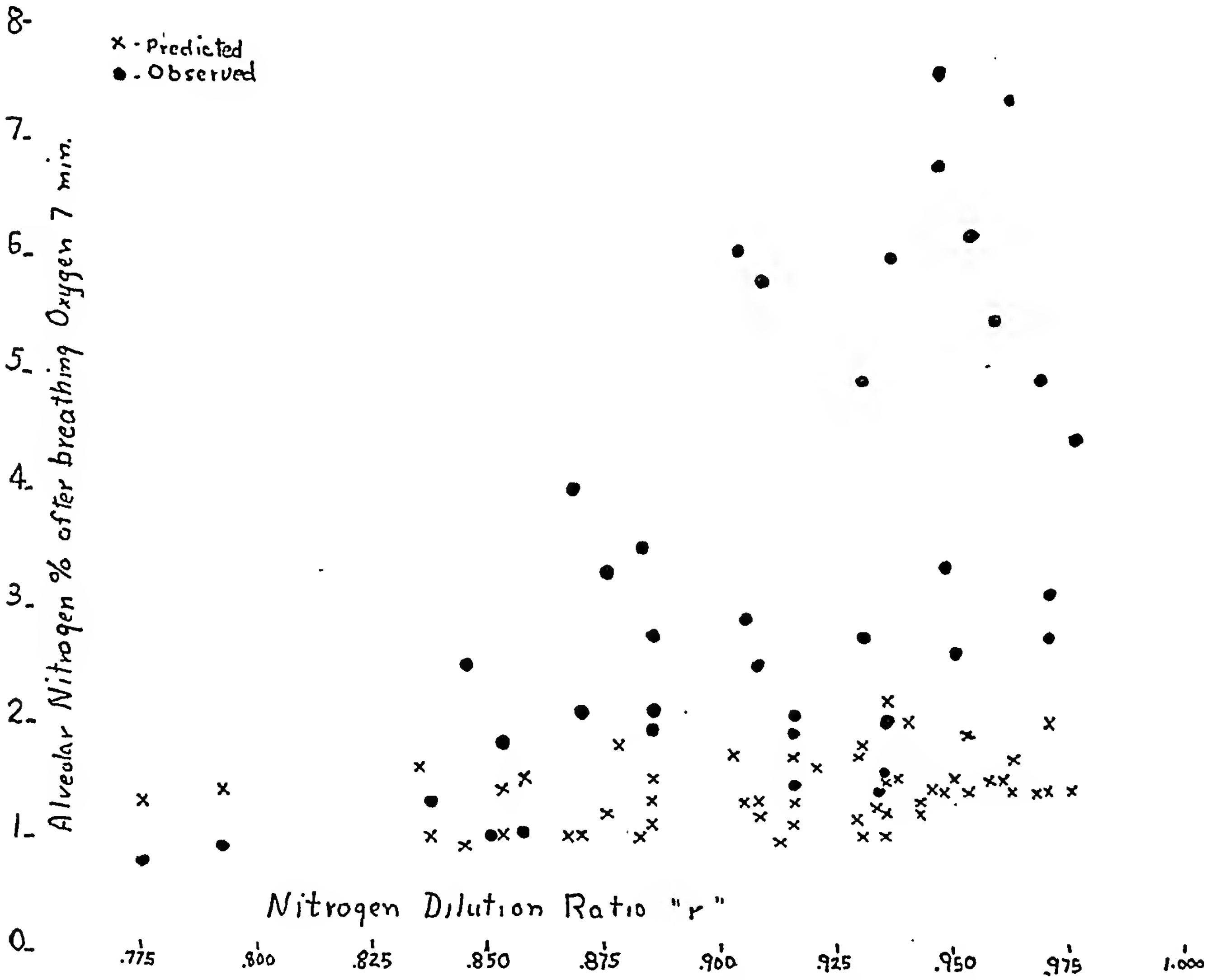


FIG. 2. THE NITROGEN CONCENTRATION OF ALVEOLAR AIR AFTER SEVEN MINUTES OF BREATHING OXYGEN,[6] PLOTTED AGAINST THE RATIO OF DILUTION OF FUNCTIONAL RESIDUAL AIR WITH EACH BREATH ("r") IN PATIENTS EXPOSED TO PHOSGENE

The observed values are indicated by solid circles and the predicted values by crosses. During shallow breathing (large "r"), there are more frequent and larger deviations from the predicted concentration than during deep breathing. This figure includes observations on the patients who had been acutely exposed to phosgene and also on the patients who had been chronically exposed to phosgene (34).

[6] The concentration of alveolar air nitrogen after 7 minutes of breathing oxygen is another index of effectiveness of intrapulmonary mixing of gases. 2.5 volumes per cent is considered the upper limit of normal in the presence of a normal tidal air volume (20).

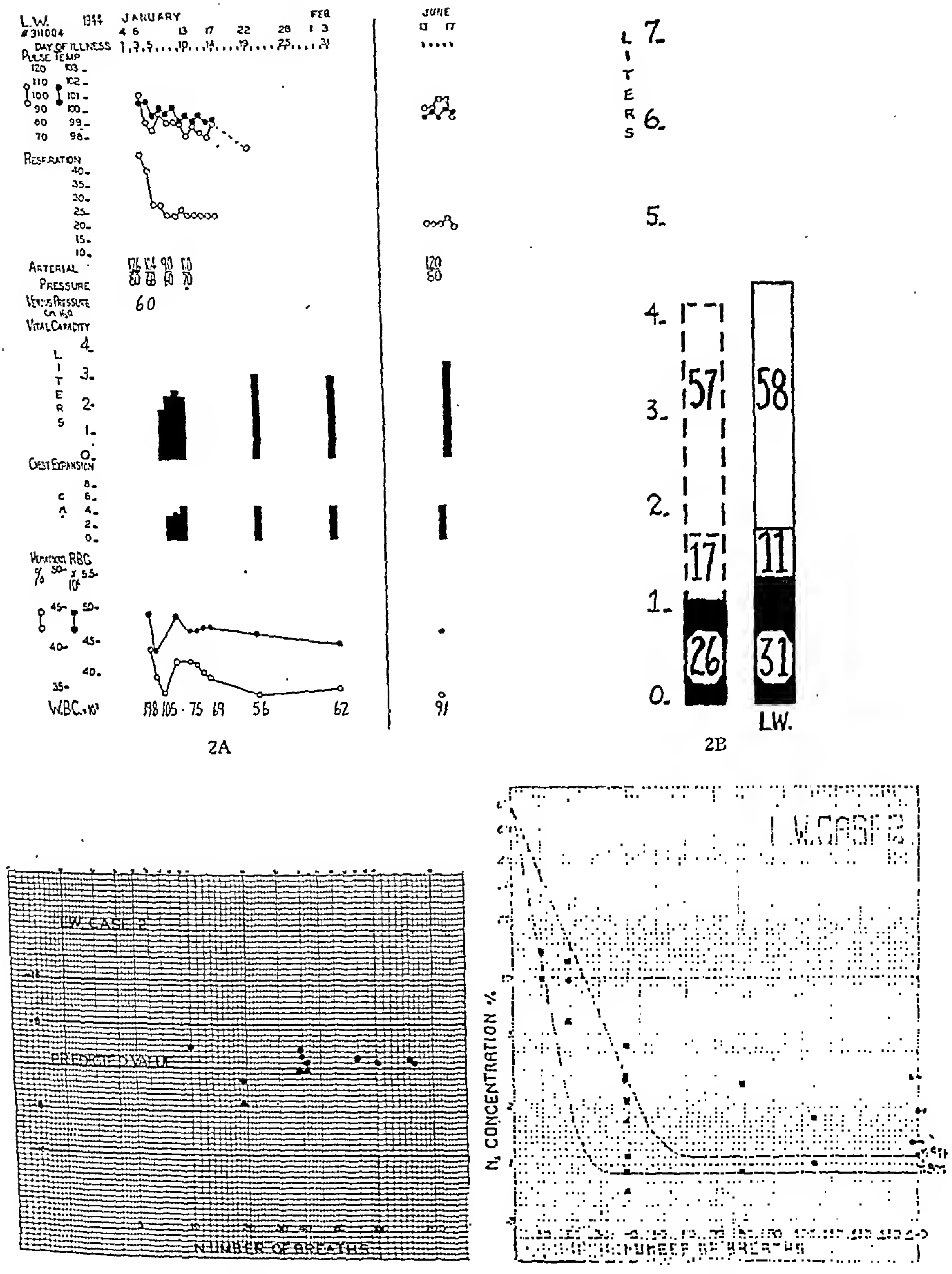


FIG. 2A. CLINICAL COURSE; 2B. SUBDIVISIONS OF LUNG VOLUME; 2C AND 2D. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 403–610 ml. (generally 475 ml.). DEAD SPACE: 227 ml.

When there is a disturbance in mixing, elimination of nitrogen from the lungs will be delayed, and calculated and observed alveolar nitrogen concentrations should exceed predicted alveolar nitrogen concentration. If there are large areas in the lungs where mixing is very poor and which contribute little or not at all to the alveolar air specimen, the calculated alveolar air nitrogen value should be greatly increased and the observed value may agree closely with the predicted value. Deviations of about 8 per cent or more in the above directions are considered indications of impaired intrapulmonary mixing of gases.

(b) Figures 1D, 2D, 3D, 4D, 5C, 6B. In these figures, the curves represent the range of values of the observed dilution ratios, *r*. When there are four curves on a figure, the two with the lowest *r* values represent the range during voluntary deep breathing; the other two, during normal quiet breathing.

Solid squares ■ represent predicted nitrogen concentrations; solid circles ●, observed nitrogen concentrations; and solid triangles ▲, calculated nitrogen concentrations. The open circles ○, squares □, and triangles △ represent similar ni-

(24) which includes the following factors: effective tidal air (tidal air minus respiratory dead space), functional residual air, amount of nitrogen released from the blood during the experiment, and number of breaths. Observed nitrogen concentration is the level obtained by analysis of an end-expiratory alveolar air sample. Calculated nitrogen concentration is the level which remains in the lungs. This is arrived at by subtracting the nitrogen in the expired air from the nitrogen in the functional residual air. The volume of nitrogen released from the blood during the measured number of breaths is taken into account.

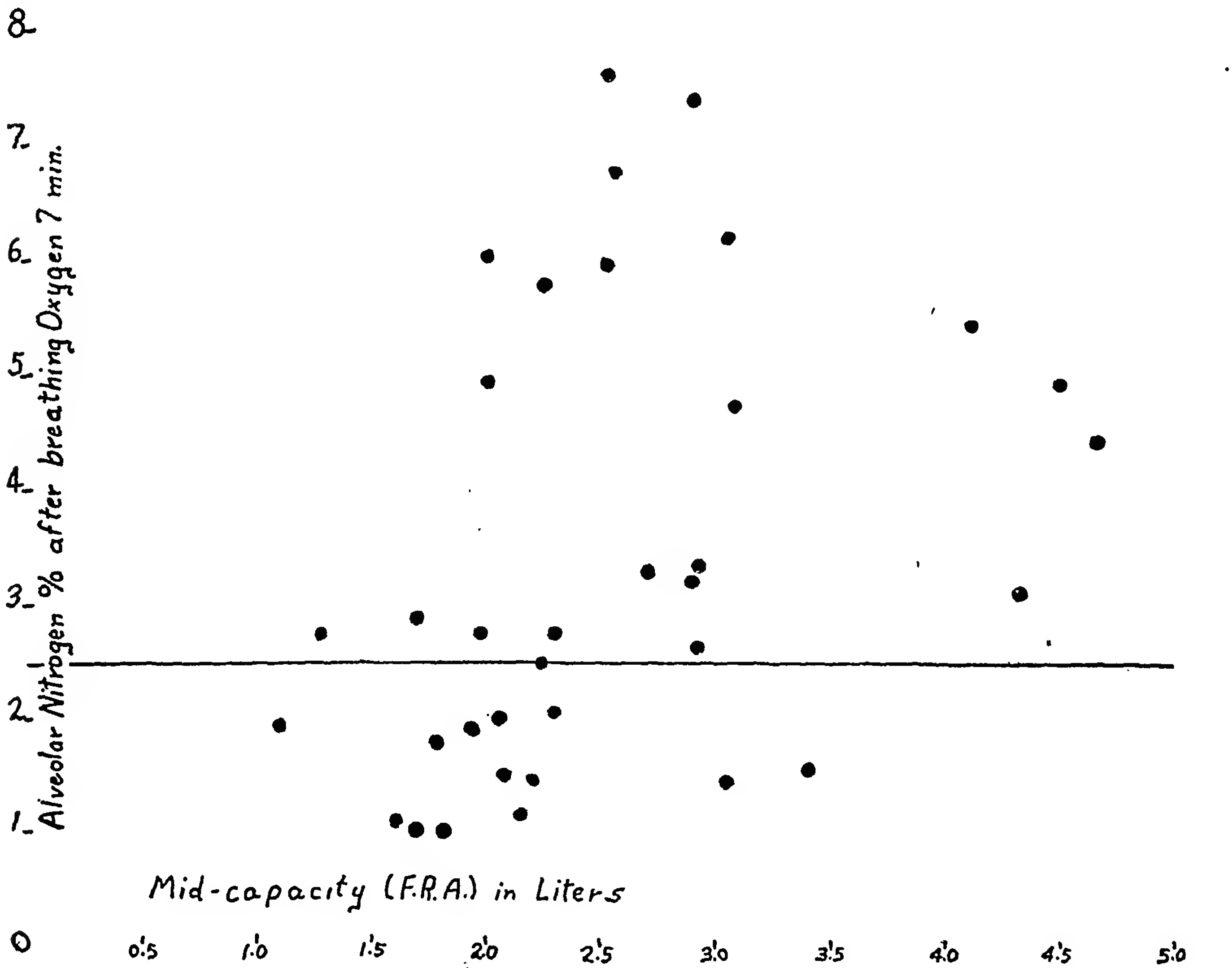


FIG. 3.

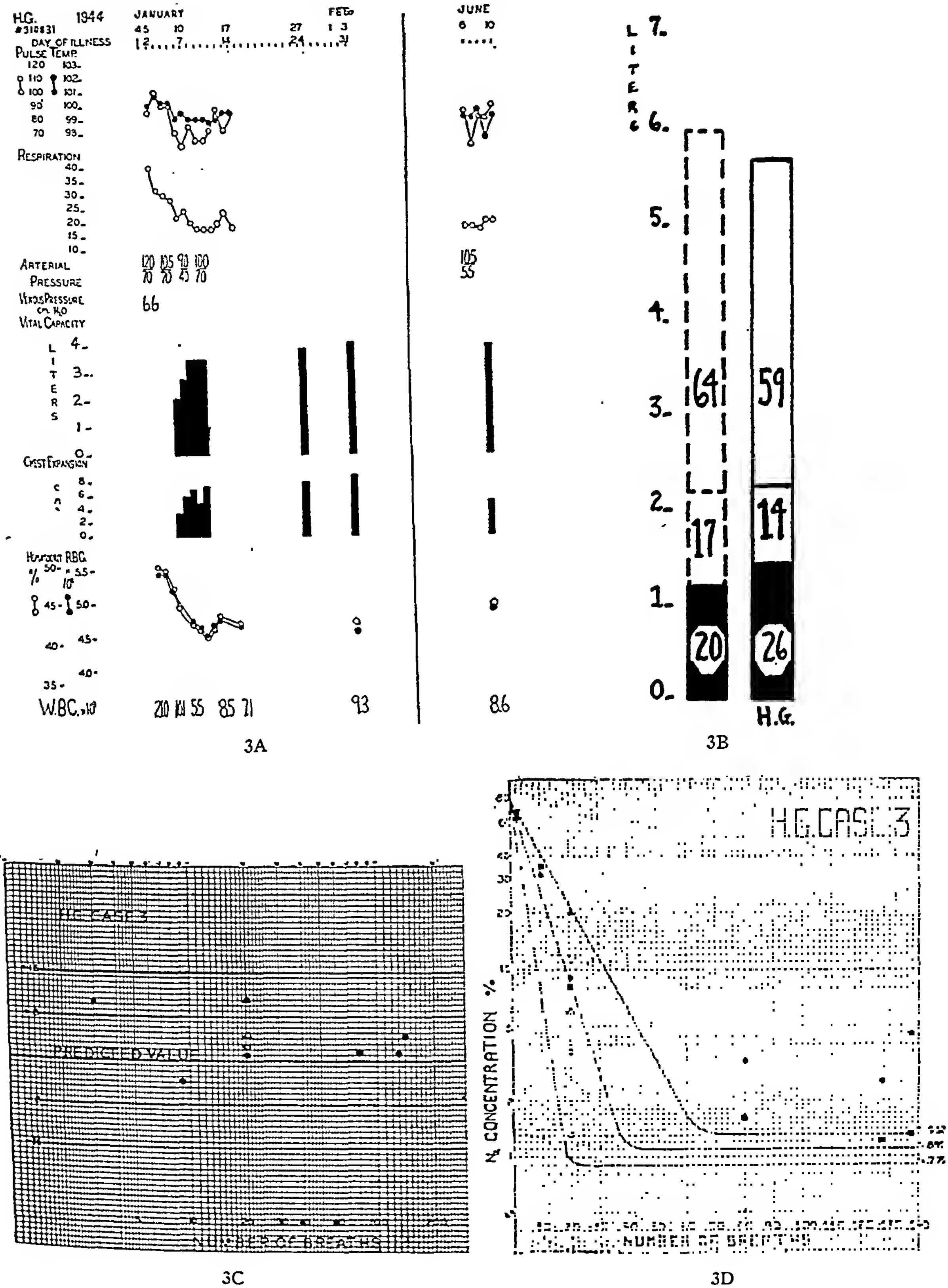


FIG. 3A. CLINICAL COURSE; 3B. SUBDIVISIONS OF LUNG VOLUME; 3C AND 3D. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 386 ml.–510 ml. (generally 400 ml.), voluntarily deepened 910 ml. DEAD SPACE: 220 ml.

trogen concentrations during voluntary deep breathing. $r =$ with a number following it, at the lower right end of each curve, represents the dilution ratio.[7]

The nitrogen concentrations marked by an arrow represent values for numbers of breaths greater than 140.

Legend for figures on subdivisions of lung volume (Figures 1B, 2B, 3B, 4B). The values obtained for each patient are compared with the normal values of Kaltrieder *et al* for individuals of the same age and sex (21) expressed in absolute volumes.

The column with initials below it represents the observed total lung volume for each patient. The column drawn in broken lines represents the predicted values for the individual (21). Each column is divided into segments which represent the per cent of the total lung volume which each subdivision comprises. The white area indicates the residual air, and the black area above it, the vital capacity. The lower segment of the vital capacity indicates the reserve air; the upper segment, the complementary air.

Legends for tables IA through VIC. In the tables dealing with exercise studies, work is expressed both as foot pounds (ft.–lb.) and kilogram meters (kgm.–m.).

In the tables dealing with arterial blood oxygen tension studies the values in parentheses under arterial blood oxygen tension represent the range of generally three, occasionally two, four, or five determinations by the direct method on the same sample of blood. The value preceding the parentheses is the average of these determinations.

Carbon dioxide tension mm. Hg arterial blood direct indicates the level of carbon dioxide tension in millimeters of mercury by the direct method (27).

Case 1 (E. T., Female, Age 38, Record No. 295042). Height 160 cm., Weight 46.3 kgm., Surface Area 1.44 M².

The patient had been working in the phosgene plant for about six months when her face was accidentally sprayed with liquid phosgene at about 10:15 a.m., June 11, 1943. Within a few hours she had developed signs of bilateral pulmonary edema and acute emphysema (Figure 1A). She was treated with oxygen inhalation under atmospheric and also positive expiratory pressure. By the third day, June 13, she had definitely improved. On the fourth day, June 14, a roentgenogram of the lungs still exhibited diffuse mottling throughout both upper lobes and the right lower lobe. On the seventh day, June 17, the lungs were clear on physical examination and a roentgenogram of the lungs appeared normal on the twelfth day, June 22.

Convalescence was complicated by complaints of aching pain in the left chest and shortness of breath on walking a few steps. In spite of these symptoms her physical condition improved and she was discharged to her home on July 3, 22 days after the accident.

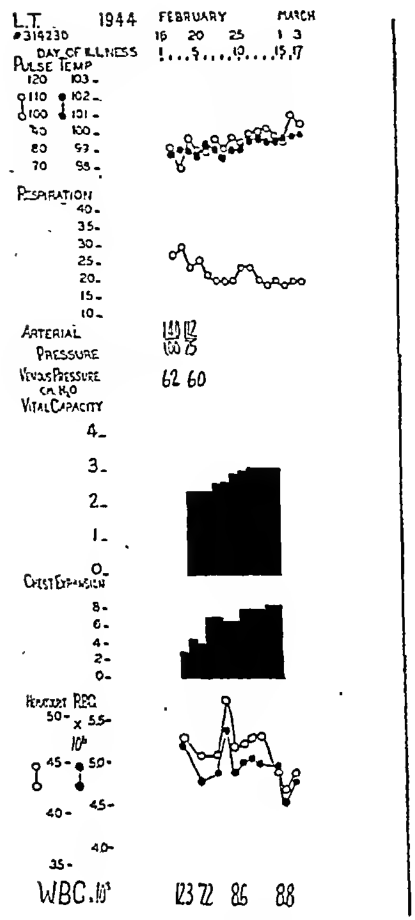
COURSE FOLLOWING FIRST ADMISSION TO THE HOSPITAL: During the nineteen months since the patient inhaled phosgene she has entered the hospital for observation on four occasions (Figure 1A).

She has complained of tiredness, weakness, marked shortness of breath, and palpitation on climbing one flight of steps, accompanied by sensation of a tight band around the lower part of the chest, weakness of the left side of the body with hyperesthesia and hyperalgesia, nervousness when amongst people, and nightmares. There has frequently been noted tenderness of the trapezius, pectoral, biceps and shoulder muscles, and over the cardiac impulse. Tolerance for moderate exercise has improved with practice. The lungs have been clear in serial teleoroentgenogram though increased aeration of the lower ⅓ of the lung fields has appeared to develop.

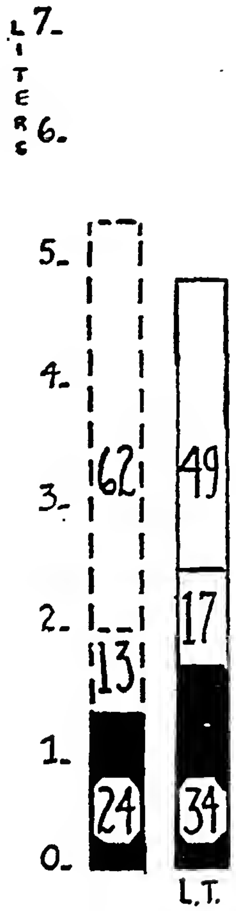
PSYCHIATRIC SUMMARY: E. T. is a rather timid, insecure, apprehensive, and not too well adjusted married white woman, who, following exposure and a rather critically sick period, developed persistent bodily aches and pains, weakness, tiredness, and palpitation, which may well have a substantial basis physically. She does have, though, a definite neurotic background, which is highlighted by interpersonal conflicts involving especially a very difficult relationship with her husband. Their sexual adjustment is quite unsatisfactory, the family relationships are not too good, and the patient finds herself annoyed and irritated by the activities of her children. Since the accident she has found it impossible to continue with her regular program of activity, she has been unable to resume work, and there has been a definite tendency toward the development of a chronic invalid reaction, which might be characterized clinically as a psychoneurosis— an anxiety state with hypochondriacal and neurasthenic features. The emotional factors have continued to assume increasing importance, and, though not recognized consciously by the patient, compensation issues are significant.

PULMONARY FUNCTION STUDIES: The ratio of residual air to total capacity is close to the upper limit of normal variation. The absolute volume of vital capacity is normal, but, because of the high residual air, the ratio of vital capacity to total capacity is reduced to the lower range of normal variation (Figure 1B).

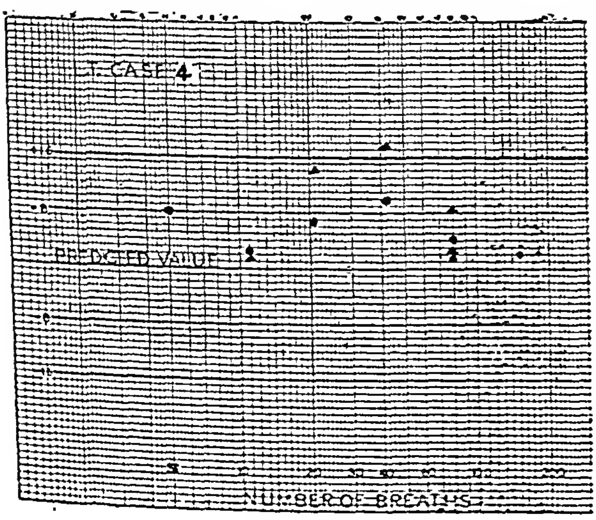
[7] The dilution ratio, r, represents the rate of dilution of nitrogen in the functional residual air per effective breath (tidal air minus respiratory dead space) of 100 per cent oxygen.



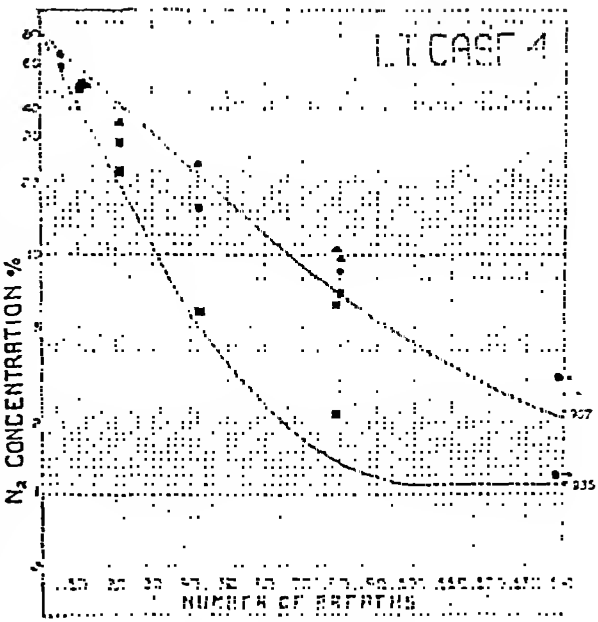
4A



4B



4C



4D

Fig. 4A. Clinical Course; 4B. Subdivisions of Lung Volume; 4C and 4D. Intrapulmonary Mixing of Gases—Tidal Air: 356–457 ml. (generally 425 ml.). Dead Space: 280 ml.

Respiratory rate and depth at rest are normal. The pattern of respiration is not altered by breathing oxygen. The minute respiratory volume during exercise increases primarily by an increase in the depth of tidal air. The alveolar air oxygen and carbon dioxide tensions are normal except for a high carbon dioxide tension occasionally (Table IA).

The nitrogen dilution rate is slow. The intrapulmonary mixing of gases is not impaired. The pulmonary emptying rate is normal (Figures 1C, 1D).

The oxygen extraction per volume of air breathed at rest is at the lower limit of normal. It rises slightly during exercise (step test) and to a greater degree during recovery. The rate of elimination of carbon dioxide is normal at rest and does not change significantly during exercise but like the oxygen uptake continues to increase during recovery (Table IA).

The arterial blood and alveolar air studies indicate that there is no disturbance in oxygen and carbon dioxide exchange in the lungs at rest and during exercise (Table IB).

The voluntary breathing capacity is 72 liters (94.0 per cent of the predicted value) when standing and 70 liters

TABLE IA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
8/14	Air	12	403	4.88	94.5	52.2	17.00	3.13
	O_2	—	—	4.93	656.0	41.0	—	—
	O_2	13.5	331	4.47	652.0	43.3	—	—
8/15	O_2	15.5	353	5.51	646.0	41.5	—	—
	O_2	14.5	440	5.89	650.0	42.0	—	—
8/16	Air	15.5	372	5.82	99.8	40.7	17.19	2.96
	Air	17	344	5.82	91.4	47.3	17.25	3.02

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
2/25	Resting	—	—	20	225	4.50	—	—
3/1	Exercise 0′–1′	4,820	666	—	—	13.10	5.88	4.04
	1′–2′	4,450	616	—	—	24.40		
	Recovery 2′–3′	—	—	—	—	28.80**	4.10	4.50
	Recovery 3′–4′	—	—	—	—	17.20		
	Recovery 4′–5′	—	—	—	—	10.00	2.15	3.07
8/19	Resting	—	—	15.5	408	6.32	3.43	2.89
	Resting	—	—	16.5	383	6.32	3.68	2.93
	Exercise 0′–1′	2,720	376	—	—	14.40	3.76	2.87
	Recovery 1′–2′	—	—	19	680	12.90		
	Recovery 2′–3′	—	—	19	600	11.35	4.48	3.86
	Recovery 3′–4′	—	—	19	492	9.35		
	Recovery 4′–5′	—	—	18	483	8.70		

* Exercise on 3/1 was on bicycle ergometer, on 8/19 was step test.

** Dyspnea at this volume.

TABLE IB

Arterial blood studies

Date 1944	State	CO_2 content	O_2 content	O_2 capacity	Saturation
		vol. per cent	*vol. per cent*	*vol. per cent*	*per cent*
2/18	Room air	—	14.9	15.5	95.6
	Oxygen	—	16.4	16.2	101.0
8/19	Room air—basal	53.0	15.7	16.2	97.0
	Room air—recovery *	47.9	16.6	17.4	95.5

Date 1945	State	Oxygen tension		Carbon dioxide tension			CO_2 content	Serum pH
		Alv. air	Art. blood	Alv. air	Arterial blood			
					Direct	Calculated		
		mm. Hg		*mm. Hg*			*vol. per cent*	
1/30	Resting	95	95(92–97)	43	41	46	57.8	7.36
1/31	Resting	96	97(96–98)	43	39	40	56.0	7.40
	After exercise 2.5 min.	123	117(116–119)	40	39	—	—	—
	5 min.	111	109(108–110)	43	37	46	42.3	7.21

* Within first minute after step test.

(101.7 per cent of the predicted value) when recumbent. Breath-holding time is normal. The results of postural tests on a tilt table are normal. The cardiac output is 1.54 liters per minute per square meter body surface area. This is a normal value.

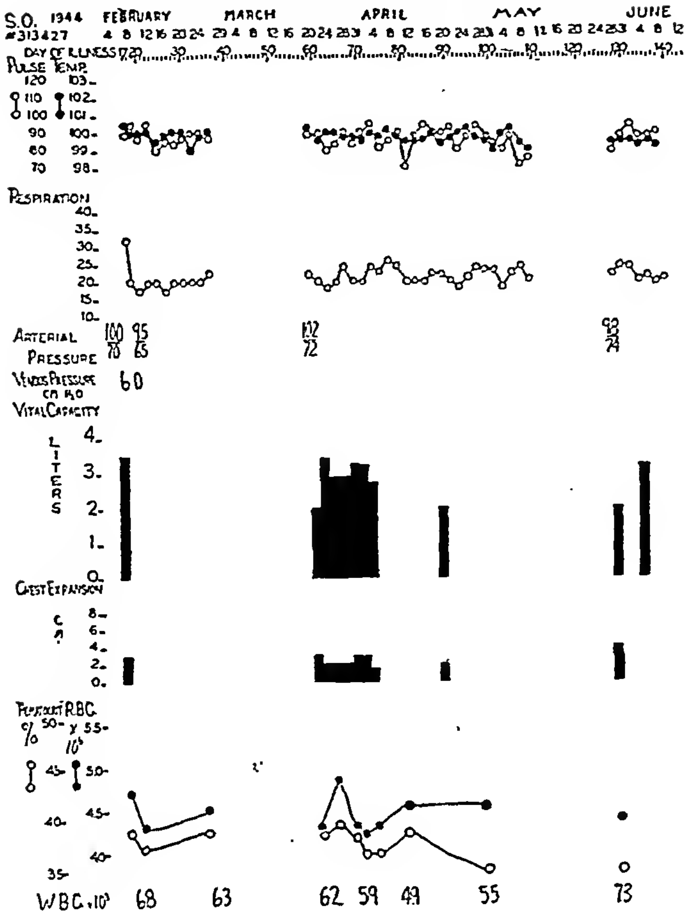
SUMMARY: A relative decrease in vital capacity and a relative increase in residual air are suggestive of early pulmonary emphysema. Ventilatory and respiratory functions of the lungs are normal.

Case 2 (L. W., Female, Age 39, Record No. 311004). Height 160 cm., Weight 61 kgm., Surface Area 1.61 M^2.

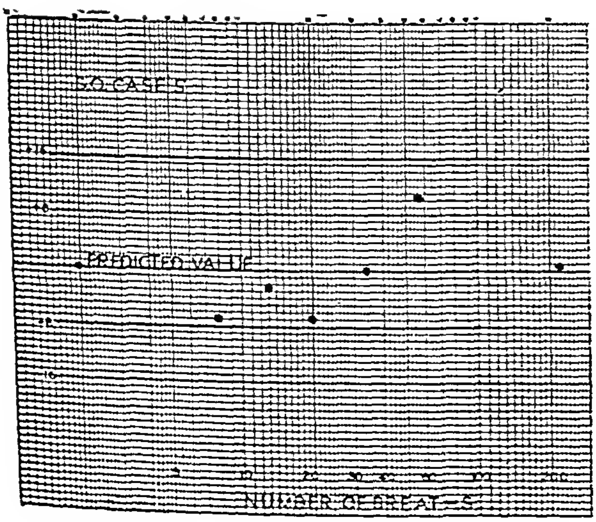
The patient had been working in the phosgene plant for about 1½ years when she accidentally inhaled phosgene at about 6:00 p.m., January 4, 1944, by placing her head under a hood in which there was a cracked glass ampule containing 40 ml. of phosgene. There were no immediate disabling symptoms and the patient worked the remainder of the shift.

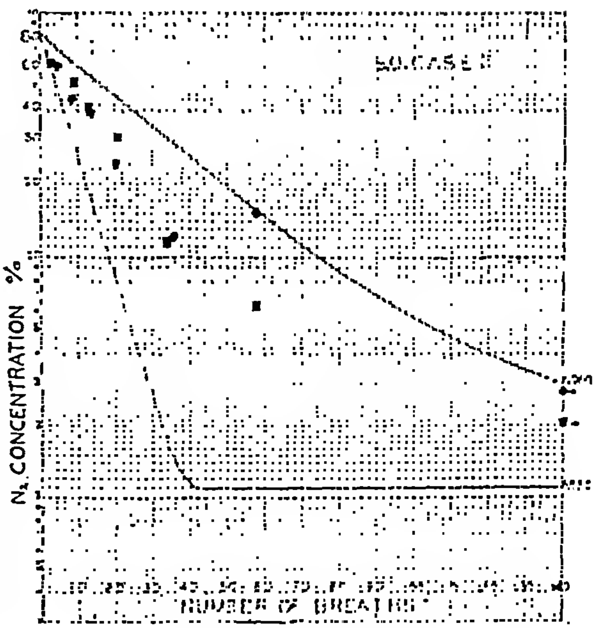
There was no history of previous inhalation of phosgene. Shortness of breath developed 6 to 8 hours after inhalation of phosgene. This symptom along with cough and expectoration of thick, yellow sputum became progressively worse during the next 1½ days.

The patient was brought to the Johns Hopkins Hospital at 12:30 p.m., January 6, 1944, approximately 41 hours after exposure to phosgene. By then there were severe pulmonary edema, acute emphysema, partial col-



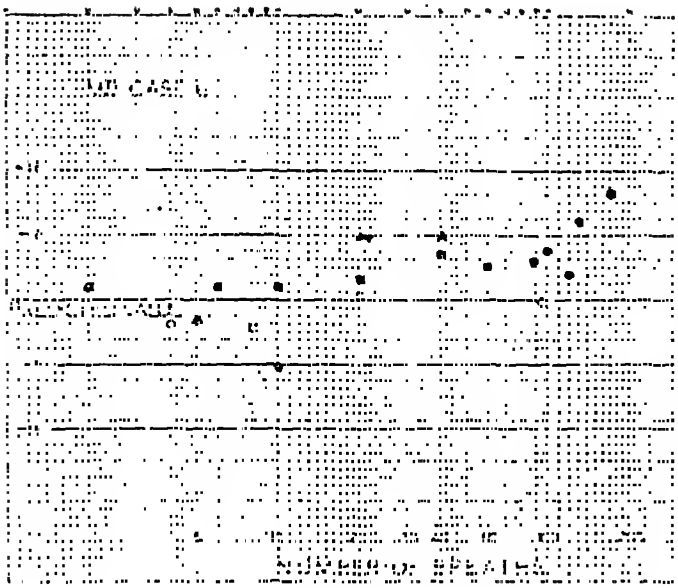
5A



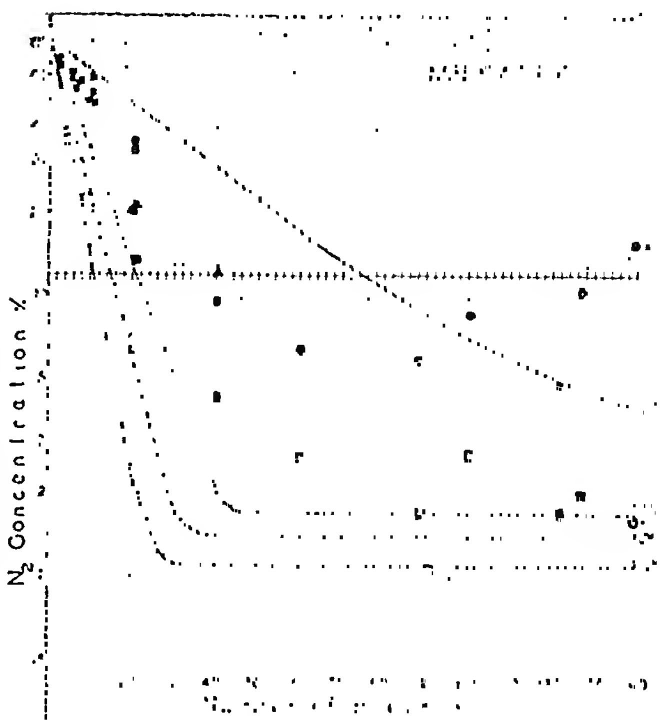


5B 5C

FIG. 5A. CLINICAL COURSE; 5B AND 5C. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 225–500 ml. (generally 325 ml.). DEAD SPACE: 165 ml. (Tidal Air below 300 ml.), 225 ml. (Tidal Air above 300 ml.)



6A



6B

FIGS. 6A AND 6B. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 220–375 ml. (generally 275 ml.), voluntarily deeped, 512–625 ml. (generally 525 ml.). DEAD SPACE: 161 ml. (Tidal Air below 400 ml.); 210 ml. (Tidal Air above 500 ml.).

lapse of the left lower lobe, marked anoxemia (arterial blood oxygen saturation 54 per cent), fever and leukocytosis (Figure 2A). Treatment consisted of continuous inhalations of 60 per cent oxygen combined with 20-minute periods of 100 per cent oxygen under 6 to 12 cm. of water expiratory pressure.

By the morning of the third day of hospitalization the patient was comfortable and without oxygen, and abnormal lung signs were practically all gone by clinical and roentgenographic examinations.

Convalescence was rapid and without complications. At the time of discharge, January 17, 11 days after admission to the hospital, the patient experienced only slight shortness of breath and no undue fatigue after moderately severe exercise.

COURSE FOLLOWING FIRST ADMISSION TO THE HOSPITAL (Figure 2A): A roentgenogram of the lungs taken one week following discharge from the hospital appeared normal. The patient returned to work one month following discharge from the hospital. Shortness of breath was no longer present at that time.

When last seen in June, 1945, approximately 17 months after the inhalation of phosgene, the patient was working, had no complaints, and did not exhibit any abnormalities on physical examination.

PSYCHIATRIC SUMMARY: L. W. is a 40-year-old widow who returns to the hospital for a checkup at this time (January 25, 1945) and says she feels perfectly all right. She had her exposure in January, 1944, and after 11 days in the hospital she took 5 weeks of rest at the suggestion of Dr. Longcope. Her course since then has been quite uneventful as far as complications are concerned, and her personal adjustment has been excellent. Her previous personality structure was that of a stable, conscientious, hard-working woman. Her attitude at present is excellent and her emotional reaction quite good. She has been able to work regularly and, although she occasionally has pain in the left side of her chest, she does not get particularly distressed by it, and actually handles it quite well without any pronounced neurotic exaggeration. She has had periods of despondency, which have not been severe or particularly incapacitating. Since her husband's death some 8 years ago, she has devoted her life to the raising of her son and has been working hard enough to provide for him adequately. She is proud of her contribution to the war effort and national emergency through her work, the buying of bonds, and giving of blood. There is very little recreation in her life because her work takes up so much of her time, but she seems to thrive on it and derives a great deal of satisfaction from this work. In addition, she gets a substantial amount of satisfaction and emotional support from the good relationship with her son. She says, "I have no alternative —I have to work to support my son."

In general, she seems a quite stable individual who has made a very good personal adjustment to her traumatic experience, showing no particularly untoward emotional response, and being able to maintain a very sensible attitude.

PULMONARY FUNCTION STUDIES: The vital capacity and the relative values of the subdivisions of the total lung volume are normal (Figure 2B).

Respiratory rate at rest is rapid, tidal air is normal, and minute respiratory volume consistently high. Breathing oxygen does not alter the pattern of respiration. During exercise, hyperventilation is even more marked than at rest because of a large increase in tidal air (Table IIA).

The nitrogen dilution rate, intrapulmonary mixing of gases, and pulmonary emptying time are normal (Figures 2C, 2D).

The maximum breathing capacity in the erect posture is reduced. It is 67.5 liters, which is 80.5 per cent of the predicted volume.

The oxygen extraction at rest per volume of air breathed falls below the normal range. The corresponding rate of carbon dioxide output is at the lower limit of normal. During exercise they both rise to well within the normal range (Table IIA).

The alveolar air oxygen tension is consistently high and the carbon dioxide tension low. This is probably due to persistent hyperventilation and is reflected by a low resting arterial blood carbon dioxide content and tension and a relatively high pH (Table IIB).

The arterial blood oxygen tension is considerably lower than that of the alveolar air at rest and after exercise (Table IIB). The air samples are representative of alveolar air since there is close agreement between the carbon dioxide tension of the alveolar air and arterial blood. There is no anoxemia. Breath-holding time is normal.

The results of postural tests on a tilt table are normal. The cardiac output is 1.05 liters per minute per square meter body surface area. This is low according to the standards used.

TABLE IIA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
6/14	Air	26	474	12.24	115.1	32.8	—	—
	O_2	22	535	11.93	676.0	26.6	—	2.27
6/15	Air	28	418	11.55	106.5	35.9	18.21	2.47
	O_2	30	412	12.50	—	—	—	2.11
6/16	O_2	26	403	10.54	661.0	28.4	—	2.54

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
6/15	Resting	—	—	28	413	11.55	2.72	2.47
6/17	Exercise 0′–1′	6,048	837	33	1,025	33.90	4.28	3.68
	Recovery 1′–2′	—	—	30	920	27.60	—	—

* Exercise consisted of climbing 3 flights of stairs (36 steps) in one minute; recovery period while standing.

TABLE IIB

Arterial blood studies

Date 1944	State	O_2 content	O_2 capacity	Saturation
		vol. per cent	*vol. per cent*	*per cent*
5/16	Room air	12.5	12.8	97.8
	Room air hyperventilation	12.6	12.8	98.4
	Oxygen	12.8	12.8	100.0

Date 1945	State	Oxygen tension		Carbon dioxide tension			CO_2 content	Serum pH
		Alv. air	Art. blood	Alv. air	Arterial blood			
					Direct	Calculated		
		mm. Hg		*mm. Hg*			*vol. per cent*	
1/25	Resting	117	101(100–103)	30	—	32	52.0	7.48
	Resting	117	98(96–99)	27	—	29	50.6	7.50
1/26	Resting (sitting in bed)	109	105(104–106)	35	42	41	50.2	7.33
	After exercise 1 min.*	117	102(101–103)	31	30	30	47.0	7.44
	5 min.	116	109(107–110)	31	29	30	47.0	7.45

* Alveolar air sample taken while sitting in bed.

SUMMARY: Ventilatory function is normal except for hyperventilation characterized by a rapid respiratory rate and a normal tidal air and a reduced voluntary breathing capacity. The low oxygen uptake and carbon dioxide output per volume of air breathed in the presence of a normal tidal air, together with the significantly higher oxygen tension in the alveolar air than in the arterial blood indicate a disturbance in respiratory function.

Case 3 (H. G., Male, Age 30, Record No. 310831). Height 177 cm., Weight 69.7 kgm., Surface Area 1.84 M².

The patient worked as a munitions handler at Edgewood Arsenal for approximately 2 years prior to the time he was incapacitated by phosgene. During that time he had two seemingly insignificant inhalations of phosgene. There was no history of lung disease.

On January 4, 1944, at 2:30 p.m., the patient accidentally took at least two full breaths of phosgene while filling shells with phosgene. He promptly experienced marked tightness in the chest, became nauseated, and vomited. There was prompt subjective improvement from oxygen and the patient returned to work in half an hour. He worked the remainder of the day though he experienced tightness in the chest, cough with expectoration of thick yellow sputum, and dizziness. These symptoms along with respiratory distress progressed during the night.

The patient entered the Johns Hopkins Hospital on January 5, almost 24 hours following the accident (Fig-

TABLE IIIA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air pO2	Alveolar air pCO2	Expired air O2	Expired air CO2
			ml.	*l.*			*per cent*	*per cent*
6/7	Air	22.0	477	10.55	100.4	38.7	—	2.60
	O_2	19.5	440	8.57	627.0	39.8	—	2.80
6/9	Air	22.0	416	9.10	105.0	38.7	17.88	2.56
	O_2	20.5	452	9.26	761.0	38.6	—	2.82

Exercise *

Date 1944	State	Work	Work	Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
6/9	Resting	—	—	22	413	9.10	3.05	2.56
6/10	Exercise 0′–1′	19,800	2,740	48	874	41.80**	2.33	3.54
	Recovery 1′–2′	—	—	37	950	35.20	—	—
6/10	Exercise 0′–1′	6,240	864	28	836	23.40	—	—
	Exercise 1′–2′	6,900	954	32	860	27.50	5.23	4.54
	Exercise 2′–3′	7,000	968	31	994	30.80	—	—

* On bicycle ergometer.
** Dyspnea.

ure 3A). By then there were pulmonary edema, acute emphysema, marked anoxemia (arterial blood oxygen saturation 53 per cent). The level of arterial blood pressure was 120/70. There were no signs of heart failure. Treatment consisted of continuous inhalations of 60 per cent oxygen under atmospheric pressure with 20-minute periods of 100 per cent oxygen under 6 to 12 cm. of water expiratory resistance.

By the morning of the third day, January 7th, the patient had definitely improved. On the fifth day, January 9th, he no longer required oxygen and on the sixth day, the lungs were clear. Convalescence was rapid and he was discharged on the thirteenth day, January 17, capable of performing moderately heavy work without any difficulty.

COURSE FOLLOWING FIRST ADMISSION TO THE HOSPITAL (Figure 3A): The patient returned to work as a painter at Edgewood Arsenal three weeks after discharge. He did not experience any limitation of physical ability.

When last seen in January, 1945, approximately one year after accidental exposure to phosgene, he was working steadily, was in good health, and did not exhibit any new physical findings.

PSYCHIATRIC SUMMARY: H. G. is a likeable, friendly, but very simple person. He is stable, has a good work record which he points to with pride, and he is conscientious. Though his exposure was followed by quite definite bodily distress, he experienced a very satisfactory emotional reaction and, largely as a result of his feeble-mindedness, which may well have prevented his being able to appreciate fully the seriousness of the situation, he was able to make a very good personal adjustment characterized by an agreeable, enthusiastic attitude, and an anxiousness to return to his former work to prove that he could take it.

PULMONARY FUNCTION STUDIES: The vital capacity is normal. The ratio of residual air to total capacity is normal (Figure 3B).

The rate of breathing is rapid; tidal air is at the lower limit of the normal range. The minute volume is high. The expired air carbon dioxide content is low and oxygen content high. The composition of the alveolar air is normal. During moderate exercise, depth of breathing increases and the carbon dioxide output and oxygen extraction rise (Table IIIA).

The rate of dilution of lung nitrogen is normal. The data concerning intrapulmonary mixing of gases are insufficient to draw definite conclusions, but several points are suggestive of impaired mixing (Figures 3C, 3D).

The oxygen saturation of the arterial blood at rest is 96.4 per cent (Table IIIB). Oxygen and carbon dioxide tension studies in arterial blood and alveolar air indicate that there is no disturbance in gaseous exchange in the lungs at rest and during exercise.

The voluntary breathing capacity in the erect posture is impaired. It is 81.8 liters which is only 62.7 per cent of the predicted value. Breath-holding is normal in duration.

Postural change on a tilt table produces no abnormal circulatory or respiratory response. The cardiac output is 1.41 liters per minute per square meter body surface area. This is a normal value.

SUMMARY: There is a disturbance in the ventilation as indicated by a low voluntary breathing capacity, a rapid respiratory rate, and suggestive evidence of impaired intrapulmonary mixing of gases. Respiratory function of the lungs is normal.

TABLE IIIB

Arterial blood studies

Date 1944	State	O_2 content	O_2 capacity	Saturation
		vol. per cent	*vol. per cent*	*per cent*
6/8	Resting	17.7	18.4	96.4

Date 1945	State	Oxygen tension Alv. air	Oxygen tension Art. blood	Carbon dioxide tension Alv. air	Carbon dioxide tension Arterial blood Direct	Carbon dioxide tension Arterial blood Calculated	CO_2 content	Serum pH
		mm. Hg	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*vol. per cent*	
1/12	Resting	96	96(94–98)	42	—	42	57.8	7.39
	Resting	99	99(97–102)	43	—	—	—	—
	After exercise 1 min.	125	125(122–128)	38	—	43	45.7	7.27

Case 4 (L. T., Male, Age 48, Record No. 314230). Height 174 cm., Weight 68.1 kgm., Surface Area 1.81 M².

The patient has been under the care of his private physician since 1938 because of inconstant aching pain in the left anterior chest, not related to breathing or exertion. Cause for the pain has not been found.

He worked in the chlorine and phosgene plants of Edgewood Arsenal for nearly two years before he was admitted to the Johns Hopkins Hospital following accidental inhalation of phosgene.

On November 15, 1942, the patient had a non-disabling exposure to chlorine. On December 23, 1943, he accidentally took a short breath of phosgene while loading shells in a box car. He soon had a choking sensation, coughed frequently, expectorated tenacious white phlegm, became nauseated, vomited, and felt dizzy. He did not seek medical attention and rested at home for the next 10 days. On returning to work there still were some cough, expectoration, and a sensation of tightness in the chest.

On February 16, 1944, he again accidentally took one or two breaths of phosgene. The immediate symptoms were similar to those following the first exposure but more intense. He sought medical attention for the symptoms. The next morning on reporting to work he was referred to Johns Hopkins Hospital (Figure 4A).

Here it was noted that the patient had acute pulmonary emphysema, edema of the base of each lung, and edema of the pharynx. There was no heart failure.

Treatment consisted of continuous inhalation of 60 per cent oxygen at atmospheric pressure and frequent 20-minute periods of 100 per cent oxygen with 5 cm. of water expiratory resistance.

Improvement was rapid. Graded exercises were begun on February 23rd, 6 days after admission. On discharge, March 3rd, 19 days after admission, he was able to perform moderately heavy work without respiratory distress or fatigue.

A roentgenogram of the chest taken on the day of admission exhibited slight clouding in the cardiohepatic angle. On the seventh day, roentgenograms of the lungs and sinuses appeared normal.

COURSE FOLLOWING FIRST ADMISSION TO THE HOSPITAL: The patient returned to work but was able to do only light labor because of pain over the left chest, similar to that which he had experienced for the past 6 years, tenderness to touch over the precordium, and shortness of breath on moderate exertion. These symptoms were still present when he was readmitted to the hospital on May 28, 1944, more than 3 months after the last inhalation of phosgene and again on January 10, 1945, approximately 6 months later. The only disturbance noted on physical examination was rapid, shallow abdominal breathing. The chest remained immobile except during deep inspiration when it expanded 7 cm. He was taught breathing exercises to encourage the use of his chest muscles in respiration. His symptoms did not improve during the period of hospitalization. His capacity for work appeared to be limited moderately.

PSYCHIATRIC SUMMARY: L. T. is a stable, well-adjusted colored male, who seems to have lived a very satisfactory life with a good work record and a well-rounded program of activities. In a conscientious sort of way, he has maintained wholesome personal contacts with relatives and friends which afforded him a considerable amount of emotional support and security. His reaction to the exposure was not accompanied by any remarkable emotional reaction, and his subsequent personal adjustment has remained good, though he continues to have some symptoms which he finds not too disturbing and to which he is able to adjust quite well, so that he has been able to resume work and live reasonably comfortably. Largely, it would appear, as a result of his previous excellent personality synthesis, he has been able to overcome the difficulties and to maintain a very reasonable attitude, with little tendency toward the development of an invalid reaction.

PULMONARY FUNCTION STUDIES: Vital capacity is abnormally low. The relationship of the residual air to the total lung volume is at the extreme upper limit of the normal range (Figure 4B).

The respiratory rate at rest is rapid, the tidal air, at the lower limit of the normal range, and the minute volume of respiration, abnormally high. Breathing oxygen does not alter the pattern of respiration. During exercise, the minute volume increases through depth of respiration. The rate becomes slower but is still rapid. The rate of oxygen extraction at rest is generally below the limit of normal variation, and the rate of carbon dioxide output

TABLE IVA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
5/30	Air	36	427	15.30	110.2	41.4	19.15	1.38
	O_2	34	426	14.70	—	—	—	1.27
5/31	Air	42	413	15.65	87.9	48.0	—	1.86
	O_2	30	434	15.55	—	—	—	2.21
6/3	Air	38	422	16.05	104.4	41.1	18.56	1.94
	O_2	37.5	379	13.05	—	—	—	1.98

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
3/6	Exercise 0′–1′	16,585	2,300	—	—	40.3	3.13	3.82
	Recovery 1′–2′	—	—	—	—	54.5**	4.39	3.97
3/7	Resting	—	—	24	442	10.6	3.53	2.91
	Exercise 0′–1′	3,980	550	—	—	20.9	4.57	3.08
	Recovery 1′–2′			—	—	25.0	4.36	3.53
6/1	Sitting on bicycle	—	—	30	533	15.8	—	—
	Exercise 0′–2′	21,000	2,900	23	1,040	23.5	3.33	3.51
	Recovery 2′–4′	—	—	30	1,050	31.5	2.73	3.41
	Recovery 4′–8′	—	—	27	950	26.5	2.33	2.35

* Bicycle ergometer.

** Subjective dyspnea at this level.

TABLE IVB

Arterial blood studies

Date 1944	State	CO_2 content	pH	pCO_2	O_2 content	O_2 capacity	Saturation
		vol. per cent			vol. per cent	vol. per cent	per cent
3/5	Room air	—	—	—	16.0	16.9	94.7
6/1	Room air	55.6	7.37	42	19.4	20.6	94.0

Date 1945	State	Oxygen tension		Carbon dioxide tension			CO_2 content	Serum pH
		Alv. air	Art. blood	Alv. air	Arterial blood			
					Direct	Calculated		
		mm. Hg		mm. Hg			vol. per cent	
1/10	Resting	89	82(80–84)	43	—	43	55.3	7.35
	Resting	89	89(85–91)	—	—	—	—	—
	Resting	97	95(92–100)	44	—	43	56.4	—
	After exercise 3.5 min.	123	118(117–119)	36	—	38	42.0	7.28

is below or close to the lowest extreme of the normal range (Table IVA).

The dilution rate of nitrogen when oxygen is breathed is slow. Intrapulmonary mixing is impaired and the pulmonary emptying rate is slow (Figures 4B, 4C).

The oxygen saturation of arterial blood at rest is normal (Table IVB). The arterial blood and alveolar air oxygen and carbon dioxide tension studies indicate that there is no disturbance in gaseous exchange in the lungs (Table IVB).

The voluntary breathing capacity when erect is 111.6 liters or 99.5 per cent of the predicted value. The breath-holding time in room air was 20 seconds in three efforts. The alveolar air carbon dioxide tension was 47 mm. Hg, and the oxygen tension, 65.8 mm. Hg at the breaking point.

Passive change in posture on a tilt table is accompanied by no unusual respiratory or circulatory response. The cardiac output is 1.37 liters per minute per square meter body surface area. This is a normal value.

SUMMARY: Pulmonary ventilation is impaired as indicated by a relatively reduced vital capacity and complementary air, and a relatively increased residual air and mid-capacity, impaired intrapulmonary mixing, and slow pulmonary emptying rate. These changes are consistent with pulmonary emphysema.

There is hyperventilation characterized by rapid and relatively shallow breathing. Respiratory function at rest and during exercise is adequate.

Case 5 (S. O., Female, Age 43, Record No. 313427). Height 156 cm., Weight 58.6 kgm., Surface Area 1.57 M².

The patient worked in the phosgene plant, Edgewood Arsenal, for approximately 2 months prior to accidental inhalation of a low concentration of phosgene for about 10 minutes on January 22, 1944.

She was promptly admitted to the Station Hospital, Edgewood Arsenal, where she complained of sneezing, watering of the eyes, substernal distress, and nausea and vomiting.

Her conjunctivae and pharynx were injected. The thorax was symmetrical, expansion shallow and equal. The lungs were clear throughout. The level of arterial pressure was 116/70. There were no signs of heart failure.

COURSE IN STATION HOSPITAL: Injection of the pharynx and conjunctivae cleared in a few days. Pulmonary edema did not develop. She frequently complained of shortness of breath on moderate exertion, cough, weakness, and pain over the heart. She was afebrile.

A teleoroentgenogram was interpreted as exhibiting old obliteration of the right costophrenic sulcus, chronic diffuse emphysema, and a partially calcified nodule in the right upper lobe. The heart appeared normal. When the patient was discharged on February 1, after 16 days of hospitalization, she still had the previously mentioned complaints.

SUBSEQUENT COURSE (Figure 5A): The patient was admitted to the Johns Hopkins Hospital 1 week later because of persistence of symptoms. There was a non-productive cough, obliteration of cardiac flatness by pulmonary resonance. The lungs were clear throughout.

The cough gradually diminished but her tolerance for exercise remained limited at the time of discharge, after 20 days of hospitalization.

She was readmitted to the hospital on March 21, 1944, for 19 days and again on May 29, 1944, for 11 days because of symptoms which were the same as on the first admission.

On each admission, no abnormalities were found on physical examination. She had numerous minor complaints and there often was slight tenderness to the touch in the 4th and 5th interspaces about the mid-clavicular line on the left.

Treatment with 60 per cent oxygen sometimes gave symptomatic relief. Breathing exercises and encouragement seemed the most effective treatment.

When last observed in June, 1944, approximately 6 months after the accident, there was no change in symptoms, tolerance for work and physical findings.

PSYCHIATRIC SUMMARY: S. O. is a somewhat timid, ineffectual, but conscientious person, who has reacted to her exposure by developing considerable anxiety with depressive manifestations. Her past life has been rather unhappy, she is an immature person, and depended on her family for emotional support, though she experienced very little real satisfaction in life. Through her work she hoped to emancipate and find some comfort in her more independent adjustment, but things did not turn out

as anticipated. The social transplantation, with the severing of old friendships and wholesome interpersonal contacts, did not work out so well. As a result there was a fertile field for the development of neurotic trends which developed following her psychologically traumatic experience and tended to exaggerate the bodily complaints and anxiety-like manifestations commonly seen in these people. Compensation issues, more or less unconsciously motivated, provided an impetus for the development of an invalid reaction with anxiety, hypochondriacal, and reactive depressive trends. Unfortunately, this illness has permitted the patient to develop a martyr-like attitude which serves a useful purpose as far as her personal needs are concerned so that it seems doubtful if she will be able to develop easily any real incentive to get better.

PULMONARY FUNCTION STUDIES: The components of the total lung volume are within the normal range. The data do not allow a calculation of the exact proportions (Table VA).

The rate of breathing is rapid, the depth is variable from test to test, although relatively constant during any one test. The tidal air is generally very low. The minute volume is higher than normal, especially when the tidal is larger than usual, as the patient does not compensate with a slower rate. During exercise, the depth is greatly increased and the minute volume becomes excessive even with mild exercise. Hyperventilation continues for at least 5 minutes after exercise. The expired air at rest contains little carbon dioxide and an abnormally high oxygen concentration, and a similar trend is observed during and following exercise. The alveolar air is of approximately normal composition (Table VB).

On oxygen breathing, the rate of dilution of lung nitrogen is slow, but no disturbance of mixing of gases is demonstrated (Figures 5B, 5C).

The arterial oxygen saturation is abnormally low at rest and rises during exercise and when breathing 100 per cent oxygen (Table VC).

The voluntary breathing capacity measured while standing is abnormally low. It is 55.5 liters or 69.5 per cent of the predicted value. Breath-holding is limited to 23 to 26 seconds and is not influenced by hyperventilation or oxygen breathing.

TABLE VA

Subdivision of lung volume

	Observed volume	Predicted volume	$\frac{\text{Observed}}{\text{Predicted}} \times 100$
	ml.	*ml.*	
Vital capacity (erect)	3,475	3,020	115.0
Complementary air (erect)	2,550	1,985	128.5
Reserve air (erect)	1,055	1,035	102.0
Mid-capacity (recumbent)	2,030	1,680	119.0

TABLE VB

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
4/12	O_2	21.5	265	6.64	—	—	—	2.01
4/15	O_2	17.0	413	6.94	—	—	—	2.62
4/24	O_2	34.5	225	7.82	652.0	40.4	—	1.52
6/5	Air	23.5	385	9.05	107.2	39.2	19.84	2.25
	O_2	27.5	412	10.08	661.0	33.9	—	3.54
6/6	O_2	22.0	387	8.60	660.0	40.2	—	2.26

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
2/25	Resting	—	—	—	—	5.30	—	—
2/26	Exercise 0′–1′	1,640	227	—	—	22.60	4.18	3.63
	Recovery 1′–2′	—	—	—	—	25.00	—	—
	Recovery 2′–3′	—	—	—	—	16.30		
	Recovery 3′–4′	—	—	—	—	13.35	2.79	2.80
	Recovery 4′–5′	—	—	—	—	13.60	—	—
	Recovery 5′–6′	—	—	—	—	12.05	2.06	2.03
4/26	Resting (on bicycle)	—	—	34	210	7.20	—	—
	Exercise 0′–1′	200**	—	34	1,020	34.70	—	—
	Recovery 1′–2′	—	—	32	835	26.80	—	—
4/26	Resting (on bicycle)	—	—	36	260	9.30	—	—
	Exercise 0′–1′	220**	—	35	1,240	43.30	—	—
	Recovery 1′–2′	—	—	40	812	32.50	—	—

* Bicycle ergometer.
** Work was obviously greater than measured.

TABLE VC

Arterial blood studies

Date 1944	State	O_2 content	O_2 capacity	Saturation
		vol. per cent	*vol. per cent*	*per cent*
2/14	Room air	13.7	15.0	91.3
	Oxygen	14.4	14.6	98.6
4/27	Room air	14.3	16.0	92.4
	Room air—exercise	15.7	16.4	95.6

Passive change in posture on a tilt table is not accompanied by any abnormal circulatory response. There is a moderate increase in the rate of respiration in the horizontal position and mild hyperventilation occurs after tilting in either direction.

SUMMARY: The relationship among the components of the total lung volume is normal. The abnormally low arterial blood oxygen saturation, in the absence of impaired intrapulmonary mixing of gases, indicates that the disturbed respiratory function is not related primarily to the rapid, shallow breathing.

Case 6 (M. P., Female, Age 49, Record No. 317296). Height 159 cm., Weight 56 kgm., Surface Area 1.55 M².

The patient worked in the chlorine plant, Edgewood Arsenal, during the summer of 1941. On July 15, 1941,

she accidentally inhaled several breaths of chlorine. She soon felt faint, short of breath on moderate exertion, became nauseated, vomited, and her throat felt sore. When she continued to feel "sick" the next day she was admitted to the Station Hospital, Edgewood Arsenal. She was discharged after a few hours. The symptoms persisted for the next 3 months.

She worked in the phosgene plant from January, 1942 to January 22, 1944.

During two weeks in December, 1943, she had several short exposures to phosgene which caused her to cough and vomit.

The patient was admitted to the Station Hospital, Edgewood Arsenal, on January 22, 1944, 1½ hours after she accidentally inhaled several breaths of phosgene which had escaped from leaking shells.

The patient was coughing violently, was nauseated, vomited, and had a nasal and lachrymal discharge.

She appeared ill. Respirations were 24 a minute, shallow. There was no cyanosis, orthopnea or dyspnea. The lungs were clear throughout; the pulse 96 per minute, regular. The level of arterial pressure was 120/80.

When discharged from the hospital after 5 days, January 26, she complained of soreness over the left side of the anterior aspect of the chest, slight shortness of breath on moderate exertion, and a non-productive cough. The lungs were clear and there was no limitation of excursion of the chest.

Roentgenograms of the lungs on admission and on the following day appeared normal. There was no leukocytosis, there was a moderate degree of normocytic anemia.

SUBSEQUENT COURSE: The patient has been unable to work steadily even at light clerical work because of precordial pain not necessarily related to effort, shortness of breath on moderate exertion, cough, and nausea and vomiting after meals.

She was observed in the hospital for 6½ weeks in March and April, 1944 and in January, 1945 for 2 days.

There have been no new physical findings. Roentgenograms of the gastrointestinal tract were normal. Electrocardiograms were normal. The anemia improved. Urine examinations were normal.

Treatment consisted of frequent massage to the legs and deep breathing exercises.

PSYCHIATRIC SUMMARY: M. P. is a modestly endowed individual with a simple social background, few outside interests, and limited interpersonal contacts upon which she can lean for emotional support. She is a conscientious, well-meaning person, with a good work record from which she obtains some additional satisfaction by being able to contribute to the national effort during the wartime emergency. Though she has the usual multiple somatic complaints and anxiety-like manifestations seen in these patients, she seems earnestly interested in trying to overcome her difficulties and in making a better personal adjustment. Emotionally she displays some instability with mild depressive trends, and some tearfulness. Clinically, the description might be that of a reactive depression with hypochondriacal and anxiety features found in a modestly endowed, conscientious woman, who probably has some more or less definite physiological bases for her symptoms which are, however, exaggerated by emotional factors; she has a strong tendency to flirt with a chronic invalid reaction with its potential gains. This attitude has been fostered to a certain extent by the numerous repeated examinations that had to be done for research reasons, and was enhanced in a way by the contacts with other patients who under similar circumstances seemed sicker, more incapacitated, and consequently standing a better chance for compensation, though it is doubtful if the patient was consciously aware of this aspect of her illness.

PULMONARY FUNCTION STUDIES: The vital capacity and the mid-capacity of the lungs are within the normal range (Table VIA).

The tidal air at rest is usually shallow, the respiratory rate is variable, and the minute volume, moderately high. The alveolar air carbon dioxide tension is generally slightly increased. During moderate exercise, the breathing becomes very rapid with only slight increase in tidal air (Table VIB).

TABLE VIA

Subdivisions of lung volume

	Observed volume	Predicted volume	$\frac{\text{Observed}}{\text{Predicted}} \times 100$
	ml.	*ml.*	
Vital capacity (erect)	3,200	3,130	102.0
Mid-capacity (recumbent)	2,000	1,750	114.0

TABLE VIB

Respiratory pattern

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
4/12	O_2	24.0	295	7.07	556.0	37.1	—	2.01
4/14	O_2	17.0	320	5.41	626.0	47.2	—	3.66
4/17	O_2	18.0	282	5.11	581.0	41.7	—	2.66
4/21	O_2	12.5	375	4.70	622.0	47.6	—	3.89
	Air	11.0	407	4.45	95.5	47.6	—	4.25

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*
5/6	Resting	—	—	20	300	6.0
	Exercise 0′–1′	2,000	277	45	465	20.9**
	Recovery 1′–2′	—	—	50	312	15.6
	Recovery 2′–3′	—	—	41	300	12.2
5/6	Exercise 0′–1′	4,420	612	60	340	19.2**
	Recovery 1′–2′	—	—	50	432	21.6**
	Recovery 2′–3′	—	—	42	370	15.4

* Bicycle ergometer.
** Dyspnea.

TABLE VIC

Arterial blood studies

Date 1944	State	O_2 content	O_2 capacity	Saturation
		vol. per cent	*vol. per cent*	*per cent*
5/3	Resting	16.7	16.7	100.0

Date 1945	State	Oxygen tension		Carbon dioxide tension			CO_2 content	Serum pH
		Alv. air	Art. blood	Alv. air	Arterial blood, Direct	Arterial blood, Calculated		
		mm. Hg		*mm. Hg*			*vol. per cent*	
1/9	Resting	103	104	45	44	47	60.0	7.35
	Resting	95	96(92–99)	—	—	—	—	—
	Deep breathing	101	99(94–104)	40	—	45	58.0	7.36

During oxygen breathing, the rate of dilution of alveolar nitrogen is slow. With shallow breathing, the observed nitrogen exceeds the predicted concentration especially at about 100 breaths. When the patient's breathing is slow and deep (5 per cent carbon dioxide added and voluntary control), the observed nitrogen concentration agrees closely with the predicted value. Apparently there are portions of the lungs which are poorly ventilated during *shallow breathing* but which react quite normally when depth is increased (Figures 6A, 6B).

The oxygen saturation of arterial blood at rest is normal. The arterial blood and alveolar air oxygen tension studies indicate that there is no disturbance in exchange of these gases in the lungs (Table VIC).

The voluntary breathing capacity in the erect posture is normal. It is 68.8 liters or 92.3 per cent of the expected value. Breath-holding time is normal.

The response to passive change in posture is normal. The cardiac output is 1.36 liters per minute per square meter body surface area. This is a normal value.

SUMMARY: The outstanding feature in this patient is the shallow breathing accompanied by moderately impaired intrapulmonary mixing of gases. This does not lead to anoxemia. Intrapulmonary mixing of gases is not impaired when respirations are deepened. Respiratory function of the lungs is not impaired.

BIBLIOGRAPHY

1. The symptoms and treatment of the late effects of gas poisoning, No. 3, Reports of the Chemical Warfare Medical Committee, British. April, 1918.
2. Barcroft, J., Hunt, G. H., and Dufton, D., Polycythemia after gas poisoning and the effect of oxygen administration in chambers in the treatment of chronic cases, No. 4, Reports of the Chemical Warfare Medical Committee, British. April, 1918.
3. Haldane, J. S., Meakins, J. C., and Priestley, J. G., The reflex restriction of respiration after gas poisoning, No. 5, Reports of the Chemical Warfare Medical Committee, British. April, 1918.
4. Hunt, G. H., Peters, R. A., Meakins, J. C., and Walker, T. W., Changes observed in the heart and circulation and the general after-effects of irritant gas poisoning, No. 7, Reports of the Chemical Warware Medical Committee, British. April, 1918.
5. Haldane, J. S., Meakins, J. C., and Priestley, J. G., Investigations of chronic cases of gas poisoning, No. 11, Reports of the Chemical Warfare Medical Committee, British. October, 1918.
6. Barcroft, J., Hunt, G. H., and Dufton, D., Treatment of patients suffering from effort syndrome by continuous inhalation of oxygen, No. 12, Reports of the Chemical Warfare Medical Committee, British. October, 1918.
7. Hunt, G. H., and Jones, C. P., A note on the later effects of poisoning by asphyxiating gas, No. 15, Reports of the Chemical Warfare Medical Committee, British. November, 1918.
8. Meakins, J. C., and Priestley, J. G., Report on the length of stay in hospital in the United Kingdom and the disposal of gas casualties, No. 16, Reports of the Chemical Warfare Medical Committee, British. November, 1918.
9. History of the great war based on official documents. Great Britain, 1923, 2, 520.
10. Blackmore, S., Discussion on the effects of asphyxiating gases on the respiratory system. Proc. Royal Soc. Med., 1937, 30, 1033.
11. Professor Achard and Major Flandin, quoted in: History of the great war based on official documents. Great Britain, 1923, 2, 407.
12. Berghoff, R. S., The more common gases, their effect on the respiratory tract; observations on 2,000 cases. Arch. Int. Med., 1919, 24, 678.
13. Pearce, R. G., Note on some respiratory studies made on late stages of gas poisoning. J. Lab. and Clin. Med., 1920, 5, 411.
14. Haggard, H. W., Action of irritant gases upon the respiratory tract. J. Ind. Hygiene, 1924, 5, 390.
15. Sergent, E., The respiratory sequelae of poisoning by war gases. Presse Medicale, 1925, 33, 201.
16. Gilchrist, H. L., and Matz, P. B., The residual effects of warfare gases. I. Chlorine. II. Mustard. War Dept., U. S. Government Printing Office, Washington, 1933.
17. Gilchrist, H. L., and Matz, P. B., The residual effects of warfare gases: the use of phosgene gas, with report of cases. The Medical Bulletin of the Veterans' Administration, 1933, 10, 1.
18. Maire, G., Hugonot, G., Giradin, M., and Thoral, L., Lesions of respiratory tract due to war gases. Revue du Service de Sante Militarie, 1938, 109, 745.
19. Darling, R. C., Cournand, A., and Richards, D. W., Jr., Studies on the intrapulmonary mixture of

gases. III. An open circuit method for measuring residual air. J. Clin. Invest., 1940, 19, 609.

20. Cournand, A., Baldwin, E. DeF., Darling, R. C., and Richards, D. W., Jr., Studies on intrapulmonary mixture of gases. IV. The significance of the pulmonary emptying rate and a simplified open circuit measurement of residual air. J. Clin. Invest., 1941, 20, 681.
21. Kaltreider, N. L., Fray, W. W., and Hyde, H. Van Z., The effect of age on the total pulmonary capacity and its subdivisions. Amer. Rev. Tuberc., 1938, 37, 662.
22. Robinson, S., Experimental studies of physical fitness in relation to age. Arbeitsphysiol., 1938, 10, 251.
23. Bohr, C., Ueber die lungenathmung. Skand. Arch. f. Physiol., 1891, 2, 236.
24. Darling, R. C., Cournand, A., and Richards, D. W., Jr., Studies on intrapulmonary mixture of gases. V. Forms of inadequate ventilation in normal and emphysematous lungs, analyzed by means of breathing pure oxygen. J. Clin. Invest., 1944, 23, 55.
25. Cournand, A., and Richards, D. W., Jr., Pulmonary insufficiency. I. Discussion of a physiological classification and presentation of clinical tests. Amer. Rev. Tuberc., 1941, 44, 26.
26. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry, Vol. II, Methods, Chapter VII: Gasometric methods for analysis of blood and other solutions. The Williams and Wilkins Co., Baltimore, Md., 1932.
27. Riley, R. L., Proemmel, D. D., and Franke, R. E., A direct method for determination of oxygen and carbon dioxide tensions in blood. J. Biol. Chem., 1945, 161, 621.
28. Comroe, J. H., Jr., and Dripps, R. D., The oxygen tension of arterial blood and alveolar air in normal human subjects. Amer. J. Physiol., 1944, 142, 700.
29. Riley, R. L., A direct method for the determination of carbon dioxide and oxygen tensions in blood. Fed. Proc., 1945, 4, 59.
30. Galdston, M., and Luetscher, J. A., Jr., Oxygen and carbon dioxide tensions in arterial blood and alveolar air at rest and after exercise in healthy subjects and in patients exposed to phosgene. Fed. Proc., 1946, 5, 265.
31. Peters, J. P., Studies of the carbon dioxide absorption curve of human blood. III. A further discussion of the form of the absorption curve plotted logarithmically with a convenient type of interpolation chart. J. Biol. Chem., 1923, 56, 745.
32. Cournand, A., Ranges, H. A., and Riley, R. L., Comparison of results of the normal ballistocardiogram and a direct Fick method in measuring the cardiac output in man. J. Clin. Invest., 1942, 21, 287.
33. Starr, I., and Schroeder, H. A., Ballistocardiogram. II. Normal standards, abnormalities commonly found in diseases of the heart and circulation, and their significance. J. Clin. Invest., 1940, 19, 437.
34. Galdston, M., Luetscher, J. A., Jr., Longcope, W. T., and Ballich, N. L., A study of the residual effects of phosgene poisoning in human subjects. II. After chronic exposure. J. Clin. Invest., 1947, 26, 169.
35. Christie, R. V., Some types of respiration in the neuroses. Quart. J. Med., 1935, 4, 427.
36. Caughey, J. L., Jr., Cardiovascular neurosis—a review. Psychosom. Med., 1939, 1, 311.
37. Cournand, A., Richards, D. W., Jr., and Darling, R. C., Graphic tracings of respiration in study of pulmonary disease. Amer. Rev. Tuberc., 1939, 40, 487.
38. Haldane, J. S., Meakins, J. C., and Priestley, J. G., The effects of shallow breathing. J. Physiol., 1919, 52, 433.
39. Starr, I., Ballistocardiographic studies of draftees rejected for neurocirculatory asthenia. War Med., 1944, 5, 155.

A STUDY OF THE RESIDUAL EFFECTS OF PHOSGENE POISONING IN HUMAN SUBJECTS. II. AFTER CHRONIC EXPOSURE

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INTRODUCTION

In contrast to the serious consequences of a single, heavy exposure to phosgene, distressing symptoms and disability are uncommon among workers repeatedly exposed to small amounts of phosgene over a period of years. Physical and roentgenographic examinations of such workers have revealed a relatively low incidence of disease of the lungs (1). There are no reports, as far as we are aware, of detailed studies of the function of the lungs in such individuals.

EXPERIMENTAL

Five workers, who had repeated exposures to small amounts of phosgene during the course of 1½ to 3½ years, were studied with particular emphasis on pulmonary function and cardiovascular and psychiatric status. The methods employed have been previously described (2).

RESULTS

Clinical summaries and data on pulmonary and cardiovascular function studies are presented in detail in the Appendix and summarized in Table I.

DISCUSSION

Most of the patients had experienced on several occasions transitory symptoms from inhalation of phosgene. Over a period of several months, all of these patients developed cough, shortness of breath on exertion, and pain or tightness of the chest, in varying degrees of severity. Sputum was scanty and mucoid. Symptoms improved on removal of the worker from exposure for several weeks.

Two of the patients (cases 2 and 3) exhibited well-marked physical and roentgenographic evidence of emphysema of the lungs, which was confirmed by an increased proportion of residual air, a definite impairment of intrapulmonary mixing of gases, prolonged pulmonary emptying of nitrogen, a limited voluntary breathing capacity, and a discrepancy between the tension of oxygen in alveolar air and arterial blood.

Anoxemia was not observed in these patients despite the well-marked ventilatory abnormalities.

One of these patients (case 3) had a history of attacks suggestive of bronchial asthma, and it is impossible to evaluate the effect of the repeated exposures to phosgene.

Two other patients (cases 1 and 4), who presented no clinical evidence of disease of the lungs, showed abnormalities of lung volume and intrapulmonary mixing of gases. Case 4 also had an arterial blood oxygen tension considerably below the alveolar air oxygen tension during exercise. These patients' objective abnormalities are equally as severe as those found in the presence of clinical signs of emphysema (cases 2 and 3).

One patient (case 5), whose chief complaint was shortness of breath on exertion, showed a reduction in voluntary breathing capacity without any clinical or other functional evidence of pulmonary disease.

The pattern of breathing in three patients (cases 2, 3, 4) was similar to that observed after acute exposure to phosgene (2). The rapid, shallow breathing was present at rest and after exercise and was associated with a subnormal extraction of oxygen per volume of air breathed.

The patients were generally seen by the psychiatrist on only one or two occasions. They appeared to be stable and not unduly disturbed about their symptoms. One patient (case 4) was under

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[2] Study carried out at the Johns Hopkins Hospital, Baltimore, Maryland, under a contract with the OEMcmr and a Medical Division, OC, CWS, Work Project Specification.

stress from a difficult situation at home, but this did not reflect itself in a tendency to exaggerate his symptoms.

Psychological factors appear to be of little consequence in their symptoms. This is in contrast to the patients studied following acute exposure to phosgene (2). This difference between the acutely and chronically exposed patients may be related to the stability of these latter patients, the lack of an isolated severe traumatic episode, or the less disturbing character of their symptoms. The previous personality structure and sources of emotional support in the form of wholesome personal contacts and secure environments seem to be significant factors.

CONCLUSIONS

1. Emphysema of the lungs may develop after chronic exposure to phosgene.
2. The measurable disturbances of the lungs are more striking after chronic exposure to phosgene than after recovery from a serious acute exposure.

TABLE I

Summary of clinical observations and data on studies performed

Case Number*	1	2	3	4	5
Patient's initials	W.L.	C.E.	T.C.	A.L.	I.H.
Age	32	50	24	31	26
Months worked with phosgene	42	36	30	16	30
Chronic symptoms	A	A	A	A	A
Physical signs: Acute	N	N	N	N	N
Chronic	N	B	A	N	N
Roentgenogram of chest	N	A	A	N	N
Volume: vital capacity	A	N	B	N	N
$\frac{\text{Per cent residual air}}{\text{Total capacity}}$	A	B	A	B	N
Intrapulmonary mixing of gases	A	A	A	A	N
Pulmonary emptying	N	B	A	A	N
Resting pattern of breathing: High rate	N	A	A	A	N
Low tidal air	N	A	A	B	N
High min. volume	N	A	A	A	N
Low O_2 extraction	N	N	B	A	N
Exercise pattern of breathing: High rate	N	B	B	B	N
Low tidal air	N	B	B	B	N
Low O_2 extraction	N	N	A	A	N
Arterial blood**: Rest	N	A	N	N	—
Exercise	N	N	A	A	—
Oxygen	N	—	—	—	—
Breath-holding	N	A	—	N	N
Voluntary breathing capacity	N	A	A	N	A
Postural tests	N	—	—	—	—
Cardiac output	N	A	N	N	—

A = Definitely abnormal. B = Borderline abnormal.
N = Normal. — = Not done.

* Listed in order studied.

** Arterial blood oxygen and alveolar air oxygen and carbon dioxide tension studies at rest and after exercise were performed 4 to 8 months after all other studies were completed. Symptoms, physical and roentgenographic findings were unchanged on re-examination of all available patients (all except 5) at that time.

3. The symptoms of chronic exposure to phosgene have not been disabling, in contrast to the frequent prolonged disability seen after acute exposure.

APPENDIX

Clinical summaries and data on pulmonary and circulatory function studies. Case histories of patients 1 through 5 are given below. The results of the pulmonary function studies are presented in Figures 1A, 1B, 1C through 5C and in Tables IA, IB through IVB and Table VA. The number of each figure and table corresponds to the case number listed in Table I.

Legend for figures on intrapulmonary mixing of gases. The data are presented in Figures 1B through 5B and 1C through 5C. The method of presentation is the same as previously described (2).

Legend for figures on subdivisions of lung volume (Figures 1A through 5A). Standards for comparison and manner of presentation are the same as described under methods (2).

Legend for Tables IA through VA, VB, VC, are the same as for Tables IA through VIC of Appendix in previous report (2).

Case 1 (W. L., Male, Age 32, Record No. 325055). Height 164 cm., Weight 80.7 kgm., Surface Area 1.78 M².

The patient voluntarily entered the Johns Hopkins Hospital on June 26, 1944, for study of his pulmonary function.

His past history is non-contributory.

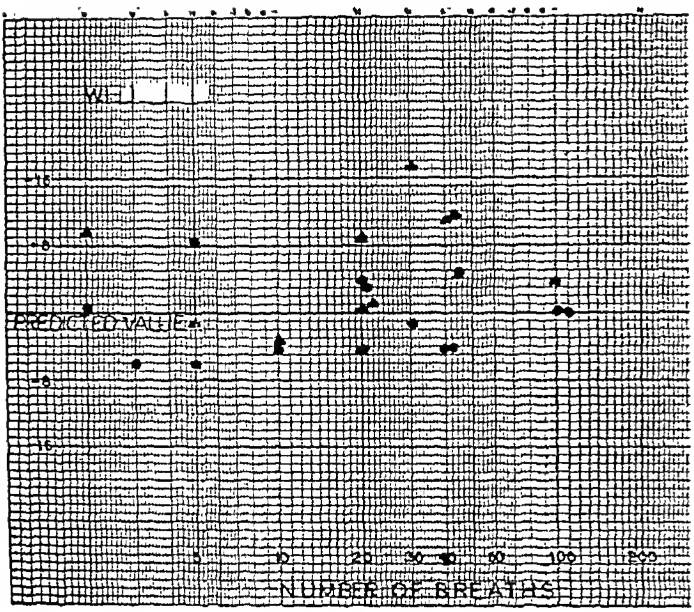
Since January 2, 1944, he has been working as a supervisor in gas warfare agent production plants of Edgewood Arsenal. He has been supervising phosgene production most of the time.

He experienced moderate conjunctivitis during the early part of 1941 and conjunctivitis and laryngitis in the fall of 1943 when he worked with mustard.

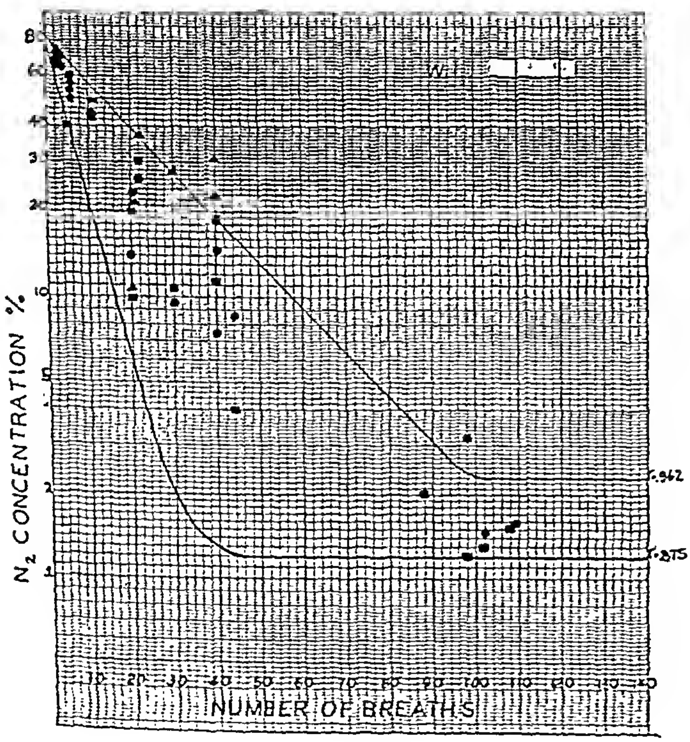
From March 6 through March 9, 1941, he accidentally inhaled small amounts of phosgene on several different occasions. This caused a sense of constriction in the chest, dizziness, mental confusion, blurred vision, and severe headaches.

On July 15, 1941, he experienced severe irritation of the throat from inhaling chlorine.

He worked steadily in the phosgene plant from October, 1943, until he entered the hospital on January 26, 1944. During the 10 days prior to entering the hospital he experienced a sensation of tightness in the chest, slight shortness of breath on exertion, and nervous twitchings in different muscles which he ascribed to recent minor exposures to phosgene.



1B



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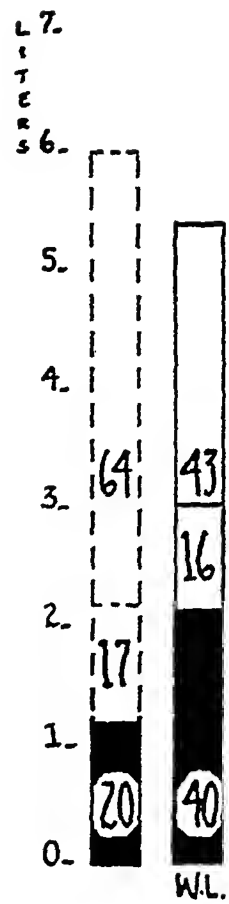


1A

FIG. 1A. SUBDIVISIONS OF LUNG VOLUME. 1B AND 1C. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 348–700 ml. (generally 500 ml.). DEAD SPACE: 265 ml.

At the time of his admission to the hospital, no abnormal findings were noted on physical examination.

A roentgenogram of the heart and lungs and an electrocardiogram were normal. The red blood count, level of hemoglobin, total and differential white blood counts, and urine were normal.

When seen again in January, 1945, approximately 6 months after the initial observation, the patient was still working in the phosgene plant. There had been no progression of symptoms. There were no new complaints or physical findings.

PULMONARY FUNCTION STUDIES: The vital capacity is reduced below the lower limit of normal variation due primarily to a low complementary air. The residual air and the mid-capacity are large and comprise abnormally high portions of the total lung volume. The ratio of complementary air to total capacity is far below the limit of normal variation (Figure 1A).

The resting respiratory rate, tidal air, and minute volume are normal. On exercise there is a marked increase in tidal air; respiratory rate does not change and the minute volume remains within the normal range. The oxygen extraction and carbon dioxide output per volume of air breathed at rest is normal, rises close to the upper limit of normal variation during exercise, and remains there during the 2 minutes immediately following exercise (Table IA).

The lung nitrogen dilution rate is slow primarily because of a large mid-capacity. Intrapulmonary mixing of gases is abnormal. Pulmonary emptying rate is normal (Figures 1B, 1C).

The arterial blood and alveolar air oxygen tension studies indicate that there is no disturbance in gaseous exchange in the lungs at rest and during exercise. There is no anoxemia (Table IB).

TABLE IA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
6/27	Air	16.5	488	8.07	94.2	43.1	17.5	2.80
	O_2	14.0	497	7.67	660.0	42.0	—	2.70
6/29	Air	13.0	535	7.03	99.1	41.4	17.3	3.30
	O_2	14.5	544	7.89	658.0	45.3	—	2.70
6/30	O_2	12.5	439	5.45	485.0	45.3	—	—
	O_2	13.5	647	8.74	510.0	36.7	—	3.05

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
6/29	Resting	—	—	13	535	7.0	3.63	3.27
	Exercise 0′–1′	—	—	12	1,238	14.8	—	—
	Recovery 1′–2′	—	—	13	1,355	17.6	7.63	5.77
	Recovery 2′–3′	—	—	13	1,254	16.3	6.03	5.47

* Bicycle ergometer.

TABLE IB

Arterial blood studies

Date 1944	State	CO_2 content	pH	pCO_2	O_2 content	O_2 capacity	Saturation
		vol. per cent			*vol. per cent*	*vol. per cent*	*per cent*
6/30	Room air	55.7	7.30	49	19.8	20.5	96.6
	Oxygen	55.1	7.35	53	20.9	20.5	102.0

Date 1945	State	Oxygen tension		Carbon dioxide tension			CO_2 content	Serum pH
		Alv. air	Art. blood	Alv. air	Arterial blood			
					Direct	Calculated		
		mm. Hg		*mm. Hg*			*vol. per cent*	
1/15	Resting	102	102(102–102)	41	—	38	57.3	7.44
1/16	After exercise 1 min.	114	110(108–113)	45	40	43	46.5	7.28

The voluntary breathing capacity in the erect posture is 127.6 liters. This is 102.6 per cent of the predicted value. The duration of breath-holding is normal.

The results of the tilt table studies are normal, except for a sustained tachycardia (100) in the erect posture. In the supine posture the pulse rate is 64 per minute. The cardiac output is 1.73 liters per minute per square meter body surface area. This is a normal value.

SUMMARY: There is impaired ventilatory function of the lungs as indicated by an absolute decrease in vital capacity, abnormally high relative volumes of mid-capacity and residual air, slow dilution rate, and impaired intrapulmonary mixing of gases. The respiratory function of the lungs is not impaired.

These findings are consistent with pulmonary emphysema.

Case 2 (C. E., Male, Age 50, Record No. 326252). Height 165 cm., Weight 77 kgm., Surface Area 1.83 M².

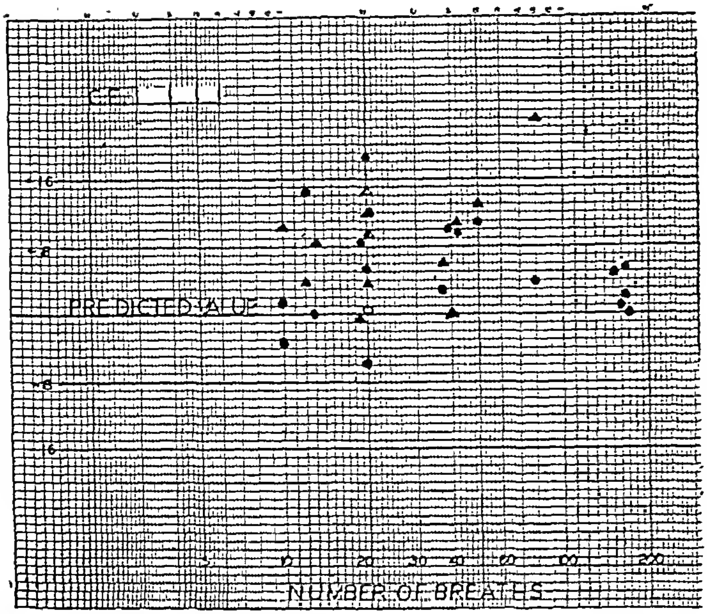
The patient voluntarily entered the Johns Hopkins Hospital on July 9, 1944, for pulmonary function studies.

His past history is non-contributory.

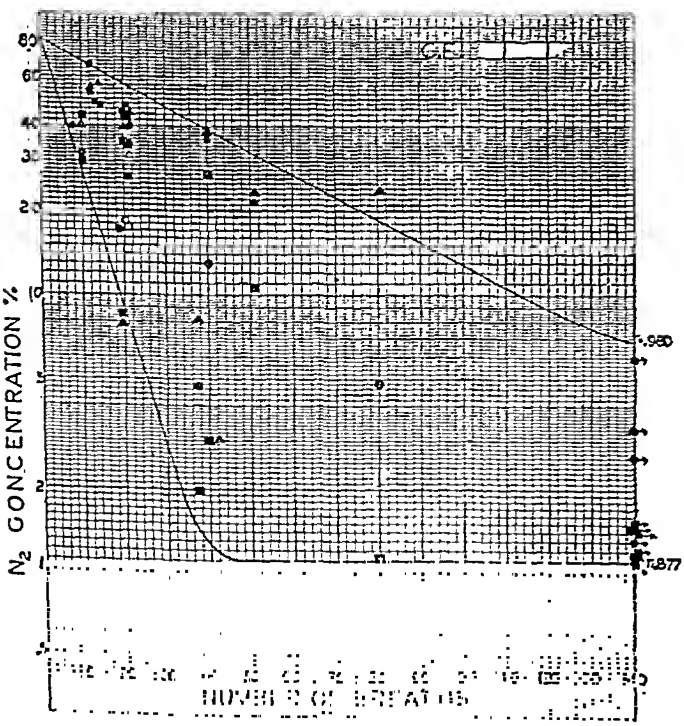
The patient has worked as a machinist in the phosgene plant, Edgewood Arsenal, since May, 1941. He has had numerous minor exposures to phosgene. These are generally followed by a sense of contriction in the throat, breathlessness, cough, nausea and vomiting of several minutes to hours duration.

He has experienced increasing shortness of breath on moderate exertion for about 3½ years, since the middle of 1941. For about one year there has been frequent cough productive of yellowish, thick sputum, which occasionally tastes of phosgene and has an offensive odor.

At the time of his admission to the hospital, no abnormalities were noted on physical examination.



2B



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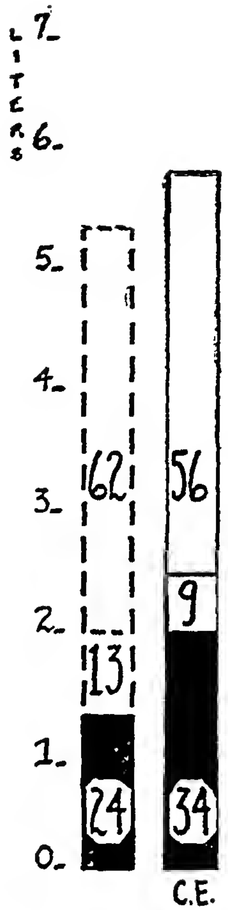


2A

FIG. 2A. SUBDIVISIONS OF LUNG VOLUME. 2B AND 2C. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 213–537 ml. (generally 350 ml.), voluntarily deepened 410 ml. DEAD SPACE: 153 ml. (Tidal Air below 350 ml.), 205 ml. (Tidal Air above 350 ml.).

The patient was without complaint except for an occasional cough and expectoration of small amounts of tenacious, yellow, non-foul sputum.

Pulmonary emphysema was suggested by the appearance of roentgenograms. The blood counts, urine, chemical studies, and level of venous pressure were normal.

When last seen in January, 1945, approximately 6 months after the initial examination, there were no new complaints or progression of the symptoms previously noted; physical and laboratory findings were unchanged.

PSYCHIATRIC SUMMARY: C. E. seems to be a fairly sensible, stable fellow, who returns to the hospital at this time (January 16, 1945) for follow-up study. He has no particular complaint. From the psychiatric standpoint he presents no remarkable findings. His exposures, while repeated, seem to have not been very disabling from the physical or emotional standpoint. His attitude toward these experiments is quite reasonable, he seems to have been not unduly distressed by the incidents, his emotional reaction is quite appropriate, and his personal adjustment to these events, in general, is quite good. He has been able to work regularly, has been able to adjust his life very well, and he seems happy. There seems to have been no serious personality deviation.

TABLE IIA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
7/10	Air	—	358	—	—	—	17.47	2.83
	O_2	25.0	349	8.78	—	—	—	—
	O_2	26.0	448	11.65	621.0	42.1	—	—
7/11	Air	38.5	508	19.63	—	—	—	—
	O_2	23.0	400	9.43	—	—	—	—
	O_2	27.0	356	9.66	—	—	—	2.70
7/12	Air	22.5	379	8.49	109.2	45.1	17.66	2.70
	O_2	27.5	330	9.08	—	—	—	—
	O_2	27.0	342	9.26	—	—	—	—
7/13	Air	24.5	344	8.34	77.4	60.8	—	—
	O_2	24.0	367	8.77	644.0	42.4	—	2.07
	O_2	22.0	269	5.88	493.0	45.1	—	2.30
	O_2	22.0	361	7.94	649.0	42.0	—	2.97
7/14	Air	20.5	393	8.09	91.2	43.5	17.60	2.88
	O_2	23.5	353	8.32	620.0	39.9	—	—
	O_2	21.5	381	8.23	—	—	—	—
7/15	O_2	25.0	361	9.07	—	—	—	—

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
7/12	Resting	—	—	26.5	305	8.07	4.23	3.02
	Exercise 0′–1′	4,530	627	—	—	21.20	3.93	3.16
	Recovery 1′–2′	—	—	27.0	879	23.70	—	—
	Recovery 2′–3′	—	—	29.0	676	19.60	—	—
	Recovery 3′–4′	—	—	26.0	542	14.10	—	—
	Recovery 4′–5′	—	—	—	—	17.60	3.13	3.43

* Step test.

TABLE IIB

Arterial blood studies

Date 1944	State	O_2 content	O_2 capacity	Saturation
		vol. per cent	*vol. per cent*	*per cent*
7/12	Resting	17.4	17.2	101.0

Date 1945	State	Oxygen tension		Carbon dioxide tension			CO_2 content	Serum pH
		Alv. air	Art. blood	Alv. air	Arterial blood			
					Direct	Calculated		
		mm. Hg		*mm. Hg*			*vol. per cent*	
1/17	Resting	110	96(95–98)	37	39	37	54.5	7.42
	Resting	108	95(94–95)	—	—	—	—	—
1/18	Resting	115	89(85–91)	37	39	38	54.2	7.39
	After exercise 1 min.	120	118(114–119)	42	34	34	44.1	7.37
	5 min.	115	110(109–110)	38	—	32	38.3	7.32

PULMONARY FUNCTION STUDIES: The vital capacity is normal. The volume of residual air comprises a relatively high per cent of the total lung volume (Figure 2A).

The respiratory rate during rest is rapid, tidal air is usually lower than normal, and minute volume is high. Breathing oxygen does not alter the pattern of respiration. During exercise the minute volume increases within the normal range primarily by a rise in tidal air.

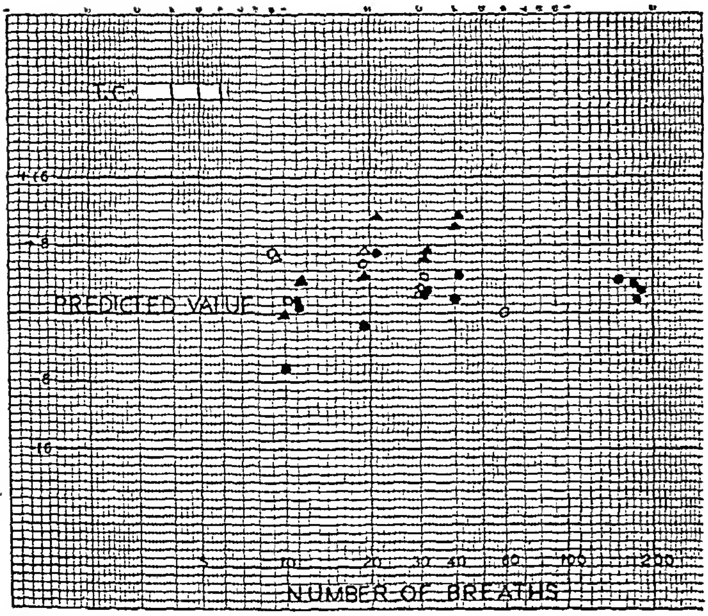
The carbon dioxide tension of alveolar air exceeds by far the normal range of variation and cannot be accounted for by breath-holding prior to collection of the alveolar sample. It would appear to be a reflection of impaired intrapulmonary mixing.

The oxygen extraction and carbon dioxide output are normal at rest, during exercise, and during recovery from exercise (Table IIA). The oxygen tension of arterial blood is significantly lower than that of the alveolar air at rest but not after strenuous exercise. There is no anoxemia (Table IIB).

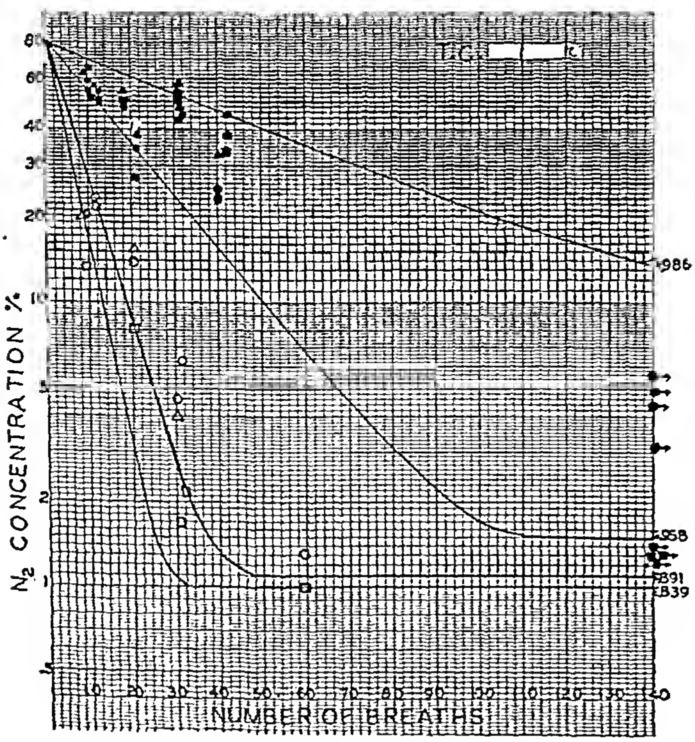
Voluntary breathing capacity in the erect posture is diminished. It is 80.6 liters or 73 per cent of the predicted value. Breath-holding time is 24 seconds in room air and 36 seconds when breathing oxygen.

The cardiac output is 0.81 liter per minute per square meter body surface area. This is an abnormally low value according to the standards used.

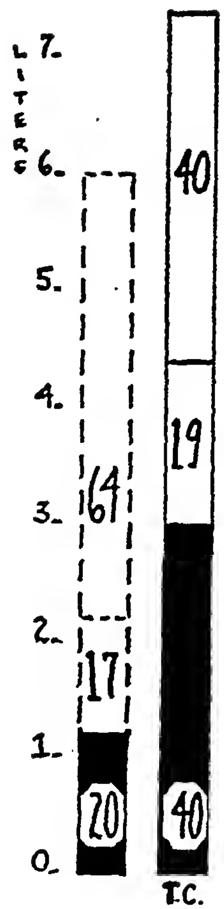
SUMMARY: The volume of residual air relative to the total lung volume is high. Intrapulmonary mixing of gases is impaired. Respirations are rapid and shallow. Maximum breathing capacity and breath-holding time are low. The oxygen extraction per volume of air breathed is normal. The oxygen tension studies indicate an impairment in respiratory function of the lungs at rest. These results are consistent with pulmonary emphysema.



3B



3C



3A

FIG. 3A. SUBDIVISIONS OF LUNG VOLUME. 3B AND 3C. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 219-448 ml. (generally 325 ml.), voluntarily deepened 709–1066 ml. (generally 850 ml.) DEAD SPACE: 205 ml.

TABLE IIIA

Respiratory pattern

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air		Expired air	
					pO_2	pCO_2	O_2	CO_2
			ml.	*l.*			*per cent*	*per cent*
7/17	Air	28.5	342	9.76	92.4	39.5	17.75	2.43
	O_2	21.5	356	7.73	630.0	45.2	—	—
	O_2	24.0	347	8.44	643.0	45.1	—	—
7/18	O_2	21.0	390	7.75	621.0	49.2	—	—
7/19	O_2	22.0	318	7.05	630.0	53.1	—	—

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
7/21	Resting	—	—	20.5	375	7.69	2.78	2.51
	Resting	—	—	26.5	322	8.53	2.98	2.37
	Exercise 0′–1′	3,600	498	—	—	13.20**	3.22	4.46
	Recovery 1′–2′	—	—	31.0	781	24.20	—	—
	Recovery 2′–3′	—	—	33.0	734	24.20	3.93	3.97
	Recovery 3′–4′	—	—	—	—	17.30	—	—
	Recovery 4′–5′	—	—	—	—	15.00	2.88	3.62

* Step test.
** Some expired air lost.

Case 3 (T., C., Male, Age 24, Record No. 326880). Height 180 cm., Weight 61.1 kgm., Surface Area 1.77 M².

The patient voluntarily entered the Johns Hopkins Hospital on July 16, 1944, for pulmonary function studies.

He has suffered from asthma during June and October since childhood. Subcutaneous injections of adrenalin afford prompt relief.

The patient worked in the chlorine plant during the winter of 1941 and had several minor exposures to chlorine.

He has worked in the phosgene plant since early 1942 and has at least on 6 occasions inhaled sufficient phosgene to cause him to experience immediate coughing, choking sensation, sweating, nausea and vomiting, and headache. He usually is fully recovered by the following day. He last inhaled phosgene in June, 1944, approximately 1 month prior to admission to the hospital.

Since 1942, the patient has experienced shortness of breath and dizziness on moderate exertion.

At the time of his admission to the hospital, the only positive findings noted on physical examination were kyphosis of the thoracic vertebrae and a few sonorous rales over the base of each lung.

A teleoroentgenogram showed voluminous, clear lungs, an emphysematous chest and a normal-sized heart. The red blood cell and total and differential white blood cell counts, level of hemoglobin, volume of packed red blood cells, and urine were normal.

When last examined, in January, 1945, approximately 6 months after the initial examination, the patient was still working in the phosgene plant. There were no new complaints or physical findings and the symptoms presented on the first admission were still present.

PSYCHIATRIC SUMMARY: T. C. seems to be a stable, hard working, conscientious individual of average intelligence, who is in no acute distress at the present time, January 19, 1945. He returns to the hospital for follow-up study and more careful evaluation. His exposures, though repeated, were not very serious and he was able to take them in his stride without their being particularly distressing or seriously altering his personal functioning. He is a quite mature man with a very sensible attitude toward his life's problems in general. His emotional reaction and present personal adjustment fall well within the normal range, and there seems to be little evidence that his personality structure or personal adjustment has been substantially influenced by his exposures at work.

PULMONARY FUNCTION STUDIES: The vital capacity is at the lower limit of normal predicted values. The residual air and mid-capacity volumes are abnormally high. Vital capacity and complementary air comprise an abnormally low percentage of the total lung volume and residual air and mid-capacity an abnormally high percentage (Figure 3A).

Respiration at rest when room air or oxygen is breathed is rapid and shallow. The minute volume is high. During exercise the rate of respiration increases moderately, the depth more than doubles, and the minute volume falls within the normal range (Table IIIA).

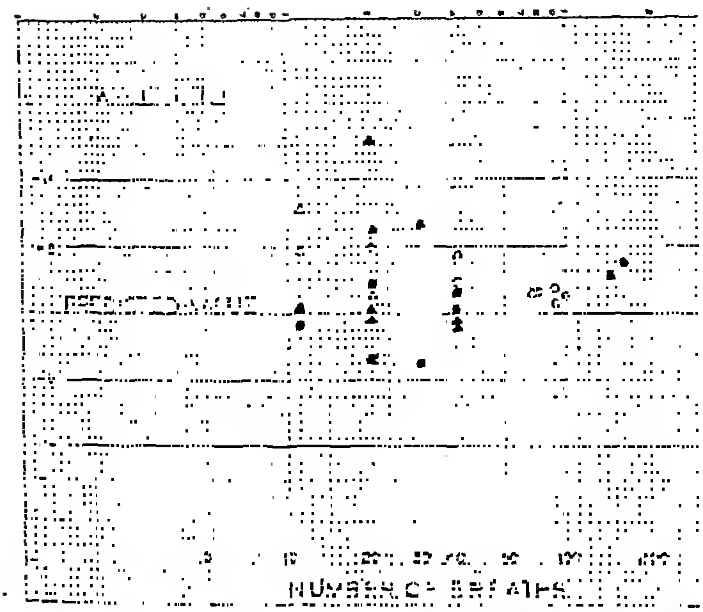
Intrapulmonary mixing of gases is impaired during quiet breathing when the dilution rate is slow and during voluntarily deepened breathing when the dilution rate is rapid. Pulmonary emptying rate is slow (Figures 3B, 3C).

TABLE IIIB

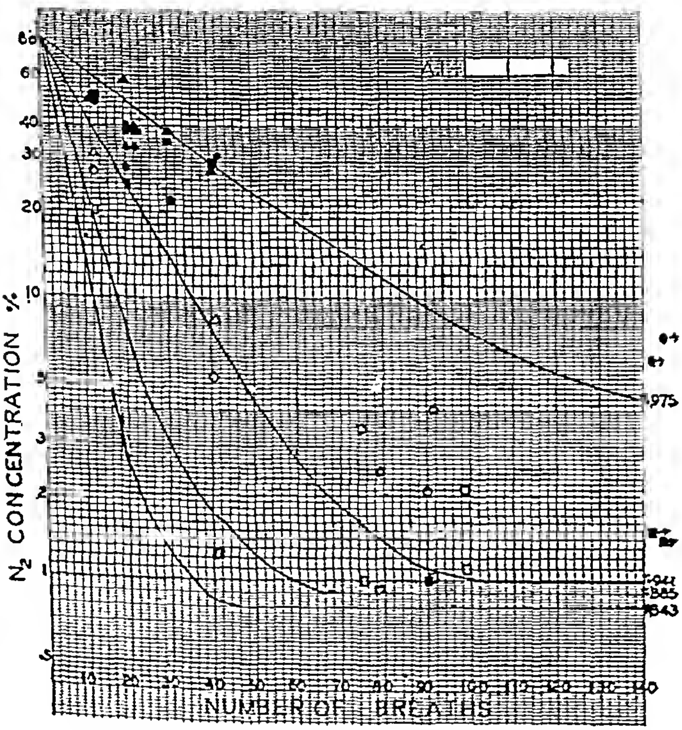
Arterial blood studies

Date 1944	State	O_2 content	O_2 capacity	Saturation
		vol. per cent	*vol. per cent*	*per cent*
7/21	Resting	20.0	21.1	95.3

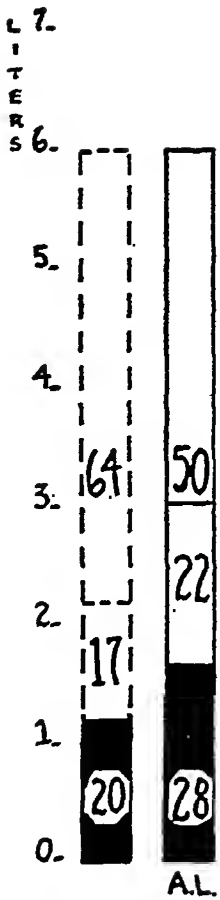
Date 1945	State	Oxygen tension		Carbon dioxide tension			CO_2 content	Serum pH
		Alv. air	Art. blood	Alv. air	Arterial blood Direct	Arterial blood Calculated		
		mm. Hg		*mm. Hg*			*vol. per cent*	
1/19	Resting	99	98(98–99)	45	44	41	58.8	7.41
	Resting	99	103(102–107)	—	—	—	—	—
1/20	After exercise							
	1 min.	124	104(102–105)	39	38	39	48.0	7.34
	5 min.	115	103(102–107)	39	—	36	47.6	7.38
	1 min. (less severe)	118	109(108–110)	43	38	—	—	—
	3 min. (less severe)	119	114(113–115)	38	43	—	—	—



4B



4C



4A

FIG. 4A. SUBDIVISIONS OF LUNG VOLUME. 4B AND 4C. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 340–460 ml. (generally 380 ml.), voluntarily deepened 669–889 ml. (generally 750 ml.). DEAD SPACE: 262 ml.

The oxygen extraction and the carbon dioxide output at rest per volume of air breathed are at the lower range of normal. During exercise, the oxygen extraction per volume of air breathed is subnormal, but carbon dioxide concentration is normal. During the first 3 minutes of recovery from exercise, the oxygen extraction rises to the normal range and during the fourth minute falls to its resting level. The carbon dioxide concentration is normal during the recovery period (Table IIIA).

These findings are consistent with the results of the arterial blood and alveolar air oxygen and carbon dioxide tension studies (Table IIIB). At rest there is no disturbance in oxygen transfer in the lungs. During strenuous exercise the alveolar air oxygen tension is approximately 20 mm. Hg greater than that of the arterial blood. This suggests the presence of a large number of inadequately circulated alveoli. There is no anoxemia.

The voluntary breathing capacity is only 63 per cent of the predicted value when measured in the erect posture. It measures 82.6 liters. The cardiac output is 1.62 liters per minute per square meter body surface area. This is a normal value.

SUMMARY: Pulmonary ventilation is definitely impaired, as indicated by a low vital capacity and a high mid-capacity and residual air, abnormally low voluntary breathing capacity, impaired intrapulmonary mixing of gases, and slow pulmonary emptying rate. Respiratory function is adequate at rest and impaired during exercise. These findings are consistent with pulmonary emphysema.

Case 4 (A. L., Male, Age 31, Record No. 309556). Height 173 cm., Weight 62.5 kgm., Surface Area 1.74 M².

The patient voluntarily entered the Johns Hopkins Hospital on August 9, 1944, for pulmonary function studies.

The patient has worked in the Production Division, Edgewood Arsenal, since 1940. In July and November, 1942, he accidentally inhaled chlorine and experienced tightness of the chest for a few days.

In the spring of 1943 there was a recurrence of pain and tightness in the chest and an episode of bronchitis, laryngitis, and aphonia following inhalation of mustard vapor.

He began to work in the phosgene plant in the fall of 1943. In March and April, 1944, and during the summer of 1944, he had some minor exposures to phosgene which caused shortness of breath on moderate exertion and sticking pain in the chest. These symptoms along with ease of fatigue, anorexia, and occasional nausea were still present when the patient entered the hospital.

In December, 1943, he was found by the Diagnostic Clinic of the Johns Hopkins Hospital to be suffering from chronic otitis media of the right ear, chronic tonsillitis and adenoiditis, and a somewhat inadequate personality makeup.

The only positive findings noted on physical examination when he entered the hospital for pulmonary function studies were perforation of the right ear drum and a few sonorous rales at the base of each lung. He expectorated one to two ounces of glairy, white, non-foul smelling sputum a day.

Roentgenograms of the lungs taken in March and April, 1944, and during observation in the hospital exhibited only an old obliteration of the left costophrenic angle.

When last seen in January, 1945, there were no new complaints or physical findings. The symptoms previously noted were still present.

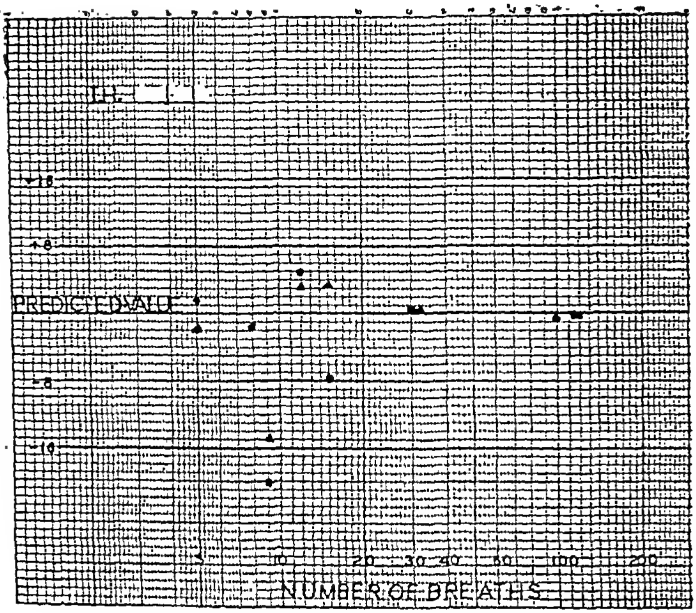
PSYCHIATRIC SUMMARY: A. L. is a man who was seen in 1943. At this time (October 20, 1944) he has no new complaints. As far as could be determined there had been no definite exposure and certainly no recent one. His desire for medical examination is, it would seem, largely related to his underlying uneasiness and general apprehension. For the most part he more or less continuously experiences symptoms of nervousness and shakiness, along with numbness, coldness, and pain over the left side of his face; these symptoms appear to be episodically exaggerated when he is under additional emotional stress. In a setting of difficult working conditions, an unresolved love affair with concern about marriage, the burden of taking care of an invalid father, and many other family responsibilities, he has grown increasingly tense, anxious, and restless. He dislikes his working conditions, is frankly frightened a great deal of the time, and experiences considerable turmoil resulting from conflict about not being in the army. It is interesting to note, though, that he apparently got some benefit from his previous interview and examination, following which he seems to have settled down some and to have been able to continue with his work Clinically, his present condition might be characterized as an anxiety state occurring in a somewhat ineffectual and inadequate personality, in a setting of emotional turmoil coincidental with his personal problems and situational stresses. While, at present, he seems to be making a neurotic adjustment, it is easily conceivable that his personality disorganization could progress to the point where it might well be thought of as a more frankly schizophrenic disorder.

PULMONARY FUNCTION STUDIES: Vital capacity is normal. The ratio of residual air and of mid-capacity to total capacity is in the upper range of normal (Figure 4A).

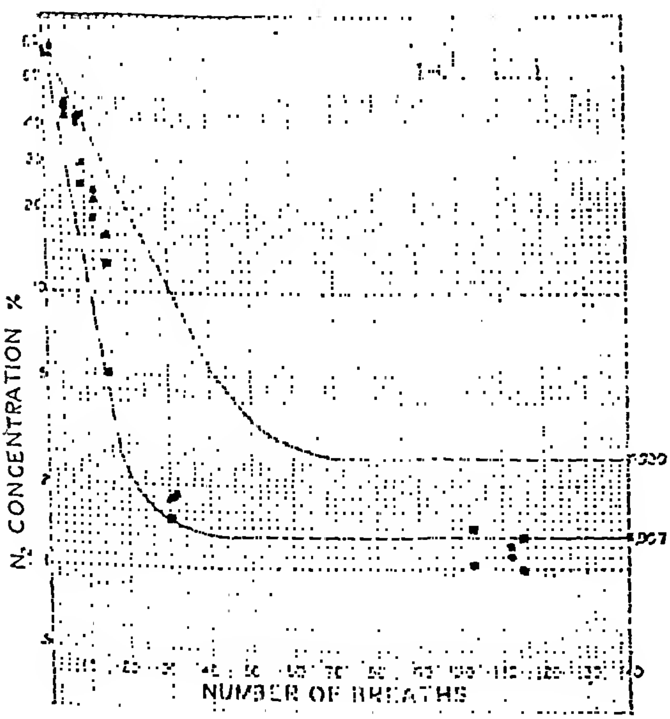
The respiratory rate at rest is rapid, the tidal air is at the lower limit of normal, and the minute volume is high. The alveolar air oxygen and carbon dioxide tensions are normal. During the exercise step test the minute volume of respiration increases within normal limits primarily due to a rise in tidal air (Table IVA).

The oxygen extraction per volume of ventilation at rest and during exercise is abnormally low. The carbon dioxide output at rest is low and during exercise and recovery is at the lower limits of normal. There is no deficiency in the oxygen uptake and the carbon dioxide output per minute because of the large minute volume of respiration (Table IVA).

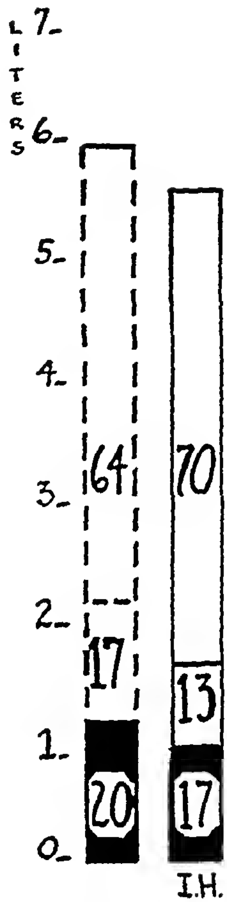
The dilution rate is slow and mixing is abnormal. Impaired mixing persists even when the dilution rate is increased by voluntary deep breathing. The pulmonary emptying rate is abnormally slow (Figures 4B, 4C).



5B



5C



5A

FIG. 5A. SUBDIVISIONS OF LUNG VOLUME. 5B AND 5C. INTRAPULMONARY MIXING OF GASES—TIDAL AIR: 233–670 ml. (generally 525 ml.). DEAD SPACE: 245 ml.

TABLE IVA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air pO₂	Alveolar air pCO₂	Expired air O₂	Expired air CO₂
			ml.	*l.*			*per cent*	*per cent*
7/31	Air	25.5	424	10.80	90.5	39.6	17.8	2.30
	O_2	23.5	406	9.50	625.0	41.8	—	—
	O_2	26	383	10.07	623.0	35.0	—	—
8/1	O_2	—	—	11.41	596.0	38.1	—	—
8/2	Air	27	488	13.14	94.0	36.0	18.5	2.39

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
8/5	Resting	—	—	20	633	12.66	2.43	2.10
	Exercise 0'–1'	3,670	509	—	—	24.80	3.13	2.80
	Recovery 1'–2'	—	—	24	985	23.60	—	—
	Recovery 2'–3'	—	—	25	808	20.30	2.93	3.08
	Recovery 3'–4'	—	—	29	594	17.20	—	—
	Recovery 4'–5'	—	—	—	—	11.00	2.31	2.58

* Step test.

The oxygen tensions of alveolar and of arterial blood are low during rest but not sufficient to cause anoxemia. During strenuous exercise, the tension studies suggest the presence of many poorly circulated alveoli since the oxygen tension of the alveolar air (5 minutes after exercise) is 13 mm. Hg greater than that of the arterial blood (Table IVB).

The voluntary breathing capacity is 110.0 liters or 89.9 per cent of the predicted volume in the erect posture. Breath-holding time is normal. The cardiac output is 1.62 liters per square meter body surface area. This is a normal value.

TABLE IVB

Arterial blood studies

Date 1945	State	Oxygen tension: Alv. air	Oxygen tension: Art. blood	Carbon dioxide tension: Alv. air	Carbon dioxide tension: Arterial blood, Direct	Carbon dioxide tension: Arterial blood, Calculated	CO_2 content	Serum pH
		mm. Hg		*mm. Hg*			*vol. per cent*	
1/23	Resting	80	79(74–83)	41	36	41	56.8	7.40
	Resting	83	83(81–85)	—	—	—	—	—
1/24	After exercise 1 min.*	93	99(98–100)	52	41	43	51.2	7.30
	5 min.	118	105(103–107)	35	31	32	45.5	7.41

* Held breath before delivered alveolar air sample. Note high alveolar air pCO_2.

SUMMARY: The residual air and mid-capacity are large in relation to the total capacity. Intrapulmonary mixing of gases is impaired and pulmonary emptying rate is slow. Voluntary breathing capacity is not reduced. Respiratory function is adequate at rest and impaired during exercise. These findings are consistent with pulmonary emphysema.

Case 5 (I. H., Male, Age 26, Record No. 330150). Height 170 cm., Weight 79.2 kgm., Surface Area 1.90 M².

The patient voluntarily entered the Johns Hopkins Hospital on August 20, 1944, for pulmonary function tests.

He worked in the phosgene plant from January, 1942 to February, 1943, in the chlorine plant from February, 1943 to November, 1943, and again in the phosgene plant from November, 1943, until admission to the hospital.

He has had a few minor exposures to phosgene which caused burning and watering of the eyes, cough, tightness in the chest, and headache. These symptoms have not at any time been disabling.

The patient has noticed moderate shortness of breath on exertion, tightness in the chest, and occasional attacks of coughing since the fall of 1943.

At the time of his admission to the hospital no abnormalities were noted on physical examination.

A roentgenogram of the lungs and heart appeared normal. There was no anemia. The total and differential white blood counts and urine were normal.

Shortly after discharge from the hospital the patient joined the Navy.

PULMONARY FUNCTION STUDIES: The relationship among the components of the total lung volume is normal (Figure 5A). The rate of breathing, tidal air, and minute

TABLE VA

Respiratory pattern resting

Date 1944	Experiment	Resp. rate	Tidal air	Min. vol.	Alveolar air pO₂	Alveolar air pCO₂	Expired air O₂	Expired air CO₂
			ml.	*l.*			*per cent*	*per cent*
8/21	O_2	14	552	7.73	669.0	37.8	—	—
	O_2	16	541	8.74	—	—	—	—
	Air	15	502	7.45	95.1	33.7	16.93	2.68
8/23	Air	13	562	7.36	92.6	43.6	16.80	3.62
	Air	13	564	7.47	79.2	42.2	16.24	3.75
	O_2	15.5	528	8.25	—	—	—	—

Exercise *

Date 1944	State	Work		Resp. rate	Tidal air	Min. vol.	O_2 extraction	CO_2 output
		ft.-lb.	*kgm.-m.*		*ml.*	*l.*	*vol. per cent*	*vol. per cent*
8/24	Resting	—	—	9	1,030	9.3	4.08	2.75
	Resting	—	—	9	982	8.9	4.78	3.86
	Exercise 0'–1'	4,670	646	—	—	29.9	4.75	3.95
	Recovery 1'–4'	—	—	—	—	17.9	4.33	4.68
	Recovery 4'–5'	—	—	—	—	11.7	3.59	4.00

* Step test.

volume are within the normal range. Alveolar and expired air are normal in composition. With exercise there is an increase in the percentage carbon dioxide output and the oxygen extraction, as well as a normal rise in minute volume of respiration (Table VA). The nitrogen dilution rate is normal and there is no disturbance of intrapulmonary mixing of gases (Figures 5B, 5C).

Voluntary breathing capacity in the erect posture is abnormally low. It is 104.2 liters or 75.2 per cent of the predicted volume. Breath-holding time is normal.

SUMMARY: Pulmonary function is normal except for a reduction in voluntary breathing capacity.

BIBLIOGRAPHY

1. Whitaker, P. H., An investigation into the radiological appearances of the chests of workers engaged in the production of toxic gases. Inclosure 1 to Military Attaché, London Report No. R3580–45 (A.3813).
2. Galdston, M., Luetscher, J. A., Jr., Longcope, W. T., and Ballich, Nicholas L., A study of the residual effects of phosgene poisoning in human subjects. I. After acute exposure. J. Clin. Invest., 1947, 26, 145.

FACTORS INFLUENCING THE URINARY EXCRETION OF CALCIUM.[1] I. IN NORMAL PERSONS

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INTRODUCTION

It is common knowledge that the urinary calcium may be markedly altered from normal in many diseases affecting calcium metabolism. The normal range of urinary calcium is very wide, and its limits have never been determined; hence, use of the amount of urinary calcium for diagnostic purposes is limited to those patients whose urinary calcium is extravagantly far from values commonly observed. It is difficult to compare the studies of one investigator made under a given set of dietary conditions with those of another investigator, using a somewhat different regimen. Some attempts to circumvent this difficulty have been made, particularly through drastic limitation of the calcium intake. The effect of the sex and size of the subject on the urinary calcium still remains in doubt.

When the person studied is still in the period of growth, the decision as to whether a given urinary calcium value is normal or abnormal becomes even more difficult. Definite demarcation of the normal range for urine calcium at all ages and intake levels should serve as a useful aid both in diagnosis and in the study of the intermediary metabolism of calcium.

THE EFFECT OF AGE, SEX, AND CALCIUM INTAKE ON URINARY CALCIUM EXCRETION BY NORMAL PERSONS

Sources of data

Even a cursory examination of data on urinary excretion of calcium discloses the wide variability of this component among normal individuals under the same conditions of study. To cite but one example, eight normal girls, 11 to 14 years old, ingested identical diets containing 1.27 grams of calcium daily; the output of calcium in the urine varied from 25 to 217 mgm. a day. With such a wide range of excretion among individuals of the same sex and of limited age range, under identical dietary conditions, it is obviously necessary to use large numbers of data in order to obtain valid conclusions as to possible relationships between the level of excretion of calcium in urine and other factors, such as calcium intake and age, sex, and skeletal size of the subject.

During the course of several years of study of calcium metabolism during growth, a considerable mass of data on urinary excretion of normal infants, children, and adults has been accumulated in this laboratory, much of which as yet is unpublished. Many of these subjects were studied at more than one level of calcium intake. To the findings from this laboratory were added data from studies of normal children and adults reported in the literature (1–44). The recently reported studies of college women (19, 26) afforded the largest contribution of data from this latter source. All studies of patients ill or convalescing from any disease whatsoever were excluded. Excluded also were studies in which the dietary regimen differed in any notable degree from the customary, also those in which the diets were markedly acid in ash. However, it was not possible, for obvious reasons, to exclude all factors known or supposed to influence urinary calcium. Milk was the major source of calcium in all diets except those containing less than 0.3 gram of calcium daily. The total calcium intake was accurately known for all subjects; all figures for calcium intake were obtained by chemical analysis of the diet. Likewise the fecal excretion and retention of calcium were known for 95 per cent of all subjects. Since the emphasis of the present study is on the excretion of calcium in the urine, and because of limitations of space, com-

[1] This paper is condensed from a part of the dissertation submitted by Elizabeth L. Knapp in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Department of Chemistry in the Graduate College of the State University of Iowa. The research was carried out in the Department of Pediatrics under the supervision of Dr. Genevieve Stearns.

TABLE I

Number and age distribution of subjects. Number of studies

Age	This laboratory		Literature		Total	
	No. subjects	No. studies	No. subjects	No. studies	No. subjects	No. studies
Infants under 1 yr.	95	880			95	880
Children 1–9 yrs.	58	112	41	51	99	163
Children 10–16 yrs.	43	104	57	59	100	163
Adults 17–80 yrs.	15	28	224	260	239	288
Total	211	1,124	322	370	533	1,494

plete data are not included, although they are available.[2] A study of the total excretion of calcium of a large number of the subjects included in this analysis has been reported in connection with other studies (19). The relative numbers and age distribution of the subjects studied are summarized in Table I.

McCrudden's method (45), or slight modifications thereof, has been used for the determination of urinary calcium in most of the studies used as source of material; therefore no serious error is introduced by combining the results from the separate studies.

[2] It is obviously impossible to include tabulations of the entire mass of data used for this study. These data are available to the investigator in the form of the thesis "Studies on Urinary Excretion of Calcium," by Elizabeth Knapp (1943), which is on file in the library of the State University of Iowa.

Urinary calcium excretion of individuals from 1 to 80 years of age

The crude relationships between the urinary calcium excretion, age, and calcium intake of the subject are shown in Table II and Figure 1. Table II gives a summary of the number of subjects, the mean values, and the standard deviations for urinary calcium excretion for each 5 years of age for all data from normal individuals from 1 to 80 years of age. In Figure 1 the mean values for four daily intake levels have been plotted and smoothed curves drawn through these points. It is evident that, although the standard deviation is always high, the mean urinary calcium excretion tends to increase both with age and with intake. At any given age the mean urinary calcium increases with the intake of calcium. In adults the amount of increase in urinary calcium with increase in intake appears to be greater than in children; hence, the spread of values becomes greater with increasing age.

The statistical validity of these assumptions was checked by analysis of several groups of data. For a study of the relationship of urinary calcium to calcium intake, it was necessary to limit the data to those studies wherein a given subject was observed with two or more levels of intake; each subject thus served as his own control. The data from 62 subjects studied in this laboratory were chosen for analysis and are summarized in Table III. Three groups, consisting of 42 boys under 12 years of age, 13 women, and 7 men, respectively, were studied at two widely differing levels

TABLE II

Summary of urinary calcium data according to calcium intake and age of subjects

Age	No. subj.	Urine Ca		No. subj.	Urine Ca		No. subj.	Urine Ca		No. subj.	Urine Ca		No. subj.	Urine Ca	
		Mean	S.D.		Mean	S.D.		Mean	S.D.		Mean	S.D.		Mean	S.D.
Ca intake *grams daily*	0–0.299			0.3–0.699			0.7–0.999			1.0–1.399			1.4 +		
years		*mgm. daily*			*mgm. daily*			*mgm. daily*			*mgm. daily*			*mgm. daily*	
1–4				13	41	13	34	47	23	42	53	31	6	62	41
5–9	8	28	21	11	71	44	31	79	39	42	96	43	19	104	59
10–14	15	76	41	18	80	46	19	94	49	48	98	55	59	117	67
15–19	3	87		17	145	53	14	178	80	25	178	71	11	237	65
20–24	36	100	48	57	118	57	34	167	68	31	181	65	12	231	53
25–29	13	107	39	22	143	52	5	167	94				9	218	52
30–34	6	121	45	6	182	67									
35–39	6	142	89	5	192	83									
40–44	2	137		2	175										
65 and over	5	147		3	196										

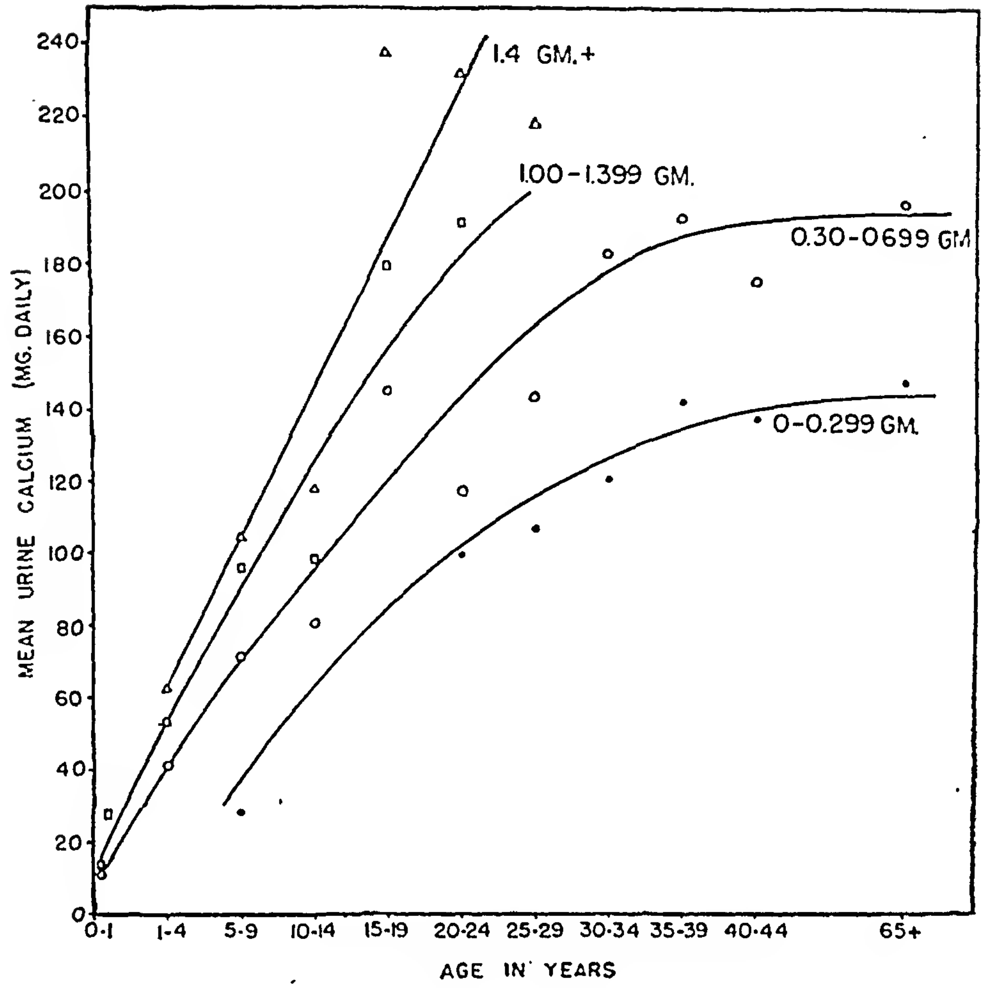

FIG. 1. THE RELATIONSHIP BETWEEN URINARY CALCIUM AND AGE OF THE SUBJECT, AT DIFFERENT LEVELS OF CALCIUM INTAKE

of intake each. Analysis showed "t" [3] to be 4.01, 5.9, and 4.6 respectively, highly significant increases in urine calcium with large increases in calcium intake.

Figure 1 also indicates that, with the daily calcium intake constant, the mean urinary calcium excretion increases with age throughout the period of growth. To determine if these differences in urinary calcium excretion at different ages were significant, the data from a group of 64 boys studied in this laboratory and ranging in age from 1 to 12 years were analyzed (Table IV). The age

TABLE III

Statistical summary of data showing range and mean urinary calcium excretion with changes in age or in calcium intake

Subjects	Number	Age range	Intake mean	Urine mean	Calcium range	Significance of differences*
		years	*grams per day*	*mgm. per day*		
Boys	42	1–12	0.554 1.446	56 81	7–224 10–260	+
Women	13	14–35	0.387 1.983	96 158	55–188 80–239	+
Men	7	20–25	0.226 2.339	131 229	81–188 130–287	+

* + = Significant at 1 per cent level.

[3] If the calculated value of "t" could occur by chance only once in 100 times, the difference is said to be "significant at the 1 per cent level," or highly significant; and if such a value could occur by chance only once in 20 times, the difference is said to be "significant at the 5 per cent level." In other words, when the values for significance are between 1 per cent and 5 per cent, there is a high probability that the differences in the means cannot be accounted for by variations due to sampling; or, as commonly expressed, the means are significantly different. Calculations of "t" were made when only 2 means were being compared, and calculations "F" when more than 2 means were compared. The standards for significance are the same for both values (75).

TABLE IV

Mean urinary calcium values of 64 boys from 1 to 12 years of age and of 23 college women. All received one quart of milk daily

	Age	Number of subjects	Calcium intake Mean	Urinary calcium Mean
	years		*grams per day*	*mgm. per day*
Boys	1	10	1.272	28
	2	7	1.267	62
	3	7	1.300	53
	4	6	1.382	79
	5	7	1.413	85
	6	5	1.397	93
	7–8	6	1.559	115
	9	5	1.574	135
	10–11	11	1.451	111
Women	17–25	23	1.378	210

range chosen covers the interval of most rapid increase of urinary calcium with age as shown in Figure 1. All boys were receiving 1 quart of milk daily in addition to the basal diet; therefore, the daily calcium intake was very nearly constant for all subjects. The number of subjects, the mean daily calcium intake, and urinary calcium for each year of age are shown in Table IV. It is obvious that although the mean calcium intake varies only about 20 to 25 per cent from 1 year to adulthood, the mean urinary calcium increases almost 10-fold during the same period. A value for "F" of 4.31 was obtained, which exceeds the value required for 1 per cent significance.

The mean urinary calcium for the 10- to 11-year old boys (Table IV) was compared with that of 23 college women receiving a similar intake (19, 26). The value of "t" was 3.94, showing that these differences also are highly significant.

It appears probable that the important factor during growth is the increase in skeletal weight of the child. In this country, skeletal weight remains approximately constant at 20 per cent of the total weight throughout life (Scammon) though the relative calcium content of the skeleton increases during growth. To test whether body weight, as a measure of skeletal weight, is the factor responsible for the change in urine calcium with age, calcium intake and urinary calcium per kgm. of body weight were calculated for all subjects for whom weights were available (606 studies). The available data were classified into 6 intake ranges and the mean, maximum, and minimum urinary calcium determined for each range (Table V). The range and mean urinary calcium values for groups 0 to 5 mgm. per kgm. and 5 to 10 mgm. per kgm. intake were so similar (Table V) that these two groups were combined, permitting a study of 148 values, representing an age range of 4 to 80 years. The mean values for urinary calcium for each 5 years of age within these groups are shown in Figure 2, compared with the mean excretion for all groups. Notwithstanding the wide range of values, the means for each age were not significantly different from the mean for the group ("F" = 1.53). A similar analysis of the possible effect of sex on excretion of urinary calcium by 67 men and 128 women showed that the differences, although significant, again were due to differences in weight of the subjects.

TABLE V

Urinary calcium per kgm. in relation to calcium intake per kgm.

Intake range	Age range	Urinary Ca Minimum	Urinary Ca Maximum	Urinary Ca Mean
mgm. per kgm. per day	*years*	*mgm. per kgm. per day*		
0–5	8–80	0.4	4.7	1.94
5–10	4–80	0.2	4.6	2.03
10–25	1–75	0.6	8.3	3.30
25–50	1–35	0.5	9.3	3.37
50–75	1–15	0.9	10.0	
75+	1–11	0.9	11.4	

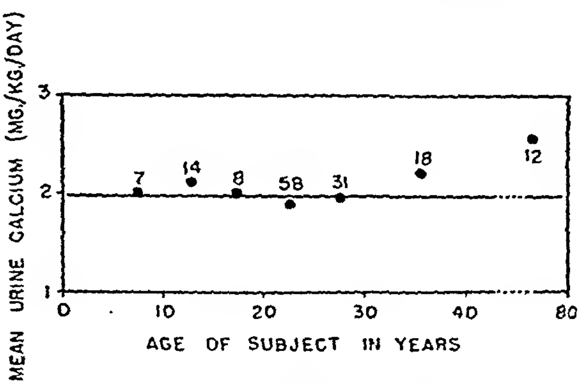

FIG. 2. THE LACK OF RELATIONSHIP BETWEEN URINARY CALCIUM, EXPRESSED IN MGM. PER KGM. DAILY, AND AGE OF SUBJECT

For this figure all data were used wherein the calcium intake was under 10 mgm. per kgm. as the range and mean urinary calcium values for groups with intakes 0–5 mgm. per kgm. and 5–10 mgm. per kgm. were almost identical (Table V). The line represents mean urinary calcium for all ages. Numbers above the dots show the number of subjects in each age group.

TABLE VI

Lack of relationship between calcium absorption and urinary excretion of calcium by children

Name	Age	Calcium			Name	Age	Calcium		
		In-take	Absorp-tion	Urine			In-take	Absorp-tion	Urine
	years	*mgm. daily*				*years*	*mgm. daily*		
R.H.	1	791 791	78 143	32 27	G.M.	2	1,331 1,331	209 328	28* 27
D.D.	1	698 698	138 281	22 27	C.C.	7	1,373 1,373	189 320	74 75*
L.R.	1	1,018 1,010	178 340	44 37	J.D.	7	1,635 1,635	702 422	46* 47*
F.S.	2	1,048 1,065	341 371	128 161	D.Br.	8	1,641 1,675	388 715	192 50*
R.B.	4	702 702	87 181	75 71	P.E.	8	1,564 1,635	404 827	248 148*
J.H.	4	685 704	68 224	63 53	P.E.	8	796 865	286 308	101 230*
F.S.	5	781 781	88 235	131 129	P.Ba.	10	1,603 1,500	531 502	155* 129*
M.D.	7	1,090 1,090	142 252	50 53	E.S.	12	1,276 1,680	117 348	34 25*
J.A.	9	809 861	305 285	94 168	V.H.	14	1,262 1,680	119 265	26 36*
M.K.	9	1,617 1,429	367 562	245 73					

* 350 I.U. of vitamin D given daily.

Skeletal weight or quantity of bone, as represented by total body weight, is thus the factor responsible for differences in urinary calcium excretion with increasing age and with sex. The relative importance of weight among the various factors affecting urinary calcium was studied by determination of the correlation coefficient, "r," between weight and urine calcium. For 45 boys 10 to 14 years old whose calcium intake was 1.0 to 1.399 grams daily, the value of "r" was 0.149; for 41 boys 10 to 14 years old with calcium intakes between 1.4 and 2.0 grams daily, "r" was 0.302. The figures required for 5 per cent significance are 0.288 and 0.304 respectively. These data tend to confirm the findings of Wang, *et al.* (43) who found no significant correlation between weight and urinary output of calcium for 23 girls, 11 to 15 years old. Thus, while urinary calcium increases significantly with increase in weight of the individual, no direct correlation can be observed between the two. Such a conclusion is to be expected if the skeletal weight is the true factor concerned. Differences in total weight due to differences in fat content of the body would not influence urinary calcium.

In studies of normal adults, McCance (23) found that the amounts of calcium in the urine varied directly with the amount of calcium absorbed and considered that "changes in the urinary excretion of calcium may be used as an index of changes in the amount of calcium absorbed." Examination of the entire group of data from this laboratory shows that in children, at least, urinary calcium level bears no relationship whatever either to calcium absorption or retention. A few illustrative examples of this lack of relationship are shown in Table VI. Apparently during the period of calcium storage, urinary calcium does not reflect the amount of calcium absorption. When storage is complete, increased urinary excretion of calcium would be expected with increased absorption of this element.

Of the factors thus far considered to affect urinary calcium, two only have been shown to be significant, calcium intake and the weight of the individual. The wide range of values obtained for individuals of the same weight and calcium intake shows that some other factor or factors peculiar to the individual are of major importance in determining the amount of calcium excreted in the urine. In an attempt to find a measure for these unknown factors, presumably endocrine, the urine calcium values were calculated as per cent of intake. The proportion of calcium intake excreted in the urine of a given person is not constant at all intake levels but is highest when the intake is low, rapidly decreasing as the intake is increased. The percentage of any given intake excreted in the urine varied widely among individuals, but all showed a decreasing percentage excreted as the intake increased. The urine calcium values calculated as per cent of calcium intake were then plotted against the calcium intake per kgm. of body weight. By using the per kgm. intake, the weight factor was eliminated and the intake factor retained. The data so compared showed a definite pattern of excretion, the percentage of intake excreted in the urine decreasing at a decreasing rate as the intake rose.

Figure 3 shows the data from 606 studies of persons 1 to 80 years old (2–44, 46, 47). The urine calcium as per cent of intake is plotted against the intake per kgm. body weight, using

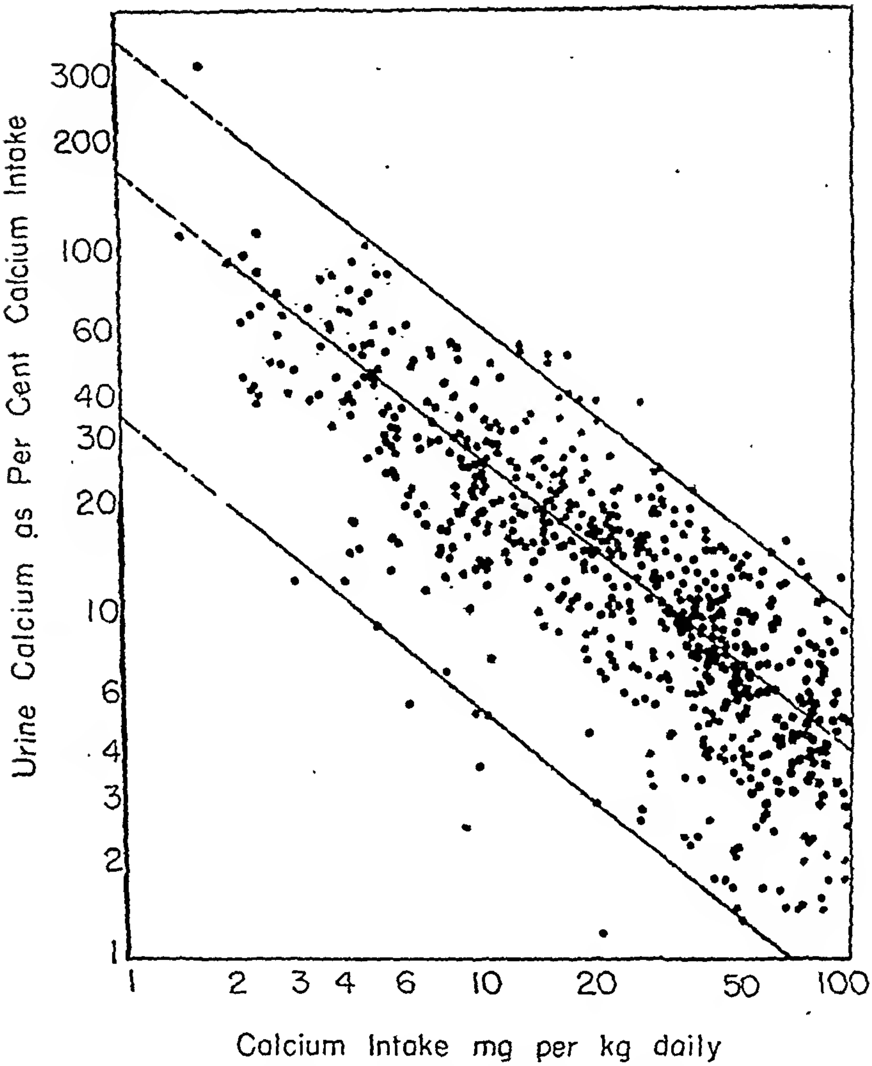

FIG. 3. THE RELATIONSHIP BETWEEN URINARY CALCIUM EXPRESSED AS PER CENT OF INTAKE, AND THE CALCIUM INTAKE IN MGM. PER KGM. DAILY

Logarithmic scales are used for both ordinate and abscissa. Mean, maximum, and minimum normal values are plotted in preference to mean and standard deviation curves. The data include 606 studies from subjects from 1 to 80 years of age (2–44, 46, 47).

a log-log scale. So plotted, it is obvious that $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ is an exponential function of the per kgm. intake. The formulas for maximum, mean, and minimum values are

$$\begin{aligned} &\text{maximum } M = 365\ I^{-.801} \\ &\text{mean } \quad y = 158.9\ I^{-.802} \\ &\text{minimum } m = 34.1\ I^{-.835} \end{aligned}$$

I being the intake per kgm. of body weight. The pertinent data for maximum, minimum, and mean lines of Figure 3 are given in Table VII. In preference to standard deviation, maximum and minimum normal values were chosen as being more useful in diagnosis of disease.

Examination of Figure 3 shows that the urinary calcium may normally be greater than the intake when the intake is less than 5 mgm. per kgm. With an intake of only 2 mgm. calcium per kgm., a urine calcium greater than 100 per cent of the intake may not be abnormal, whereas with an intake of 50 mgm. per kgm., the maximum normal excretion is 16 per cent of the intake.

TABLE VII

Urinary calcium as per cent of intake in relation to calcium intake per kilogram of body weight

Calcium intake	Urine Ca as per cent intake		
	Minimum	Mean	Maximum
mgm. per kgm. per day	*per cent*	*per cent*	*per cent*
2	19.1	91.1	210
5	8.9	43.7	101
10	5.0	25.1	58
15	3.6	18.1	42
20	2.8	14.4	33
30	2.0	10.4	24
50	1.3	6.9	16
75	0.9	5.0	12
(100)*	(0.7)	(4.0)	(9)

* Extrapolated value.

It seems probable that the position of urine calcium values of any individual person relative to the mean, as shown on Figure 3, will depend on the endocrine balance of the person. If this is true, the values for any one individual should maintain the same position relative to the mean at all levels of intake. Also, if age *per se* is not a factor, it is expected that the normal range and the mean values should be the same for all ages studied. The validity of these assumptions is shown by Figures 4 to 6. In these figures, individual data are plotted as in Figure 3. When several intake levels of calcium have been studied in one individual these values are connected by dotted lines. $\frac{\text{Urine Ca} \times 100}{\text{Intake Ca}}$ of each individual tends to maintain a constant position relative to the mean at all levels of intake. For each group, the values tend to be distributed about the mean in a manner similar to that of the entire group. The only exceptions to this rule are the values for children 1 to 2 years of age, which tend to lie almost wholly below the normal mean, though

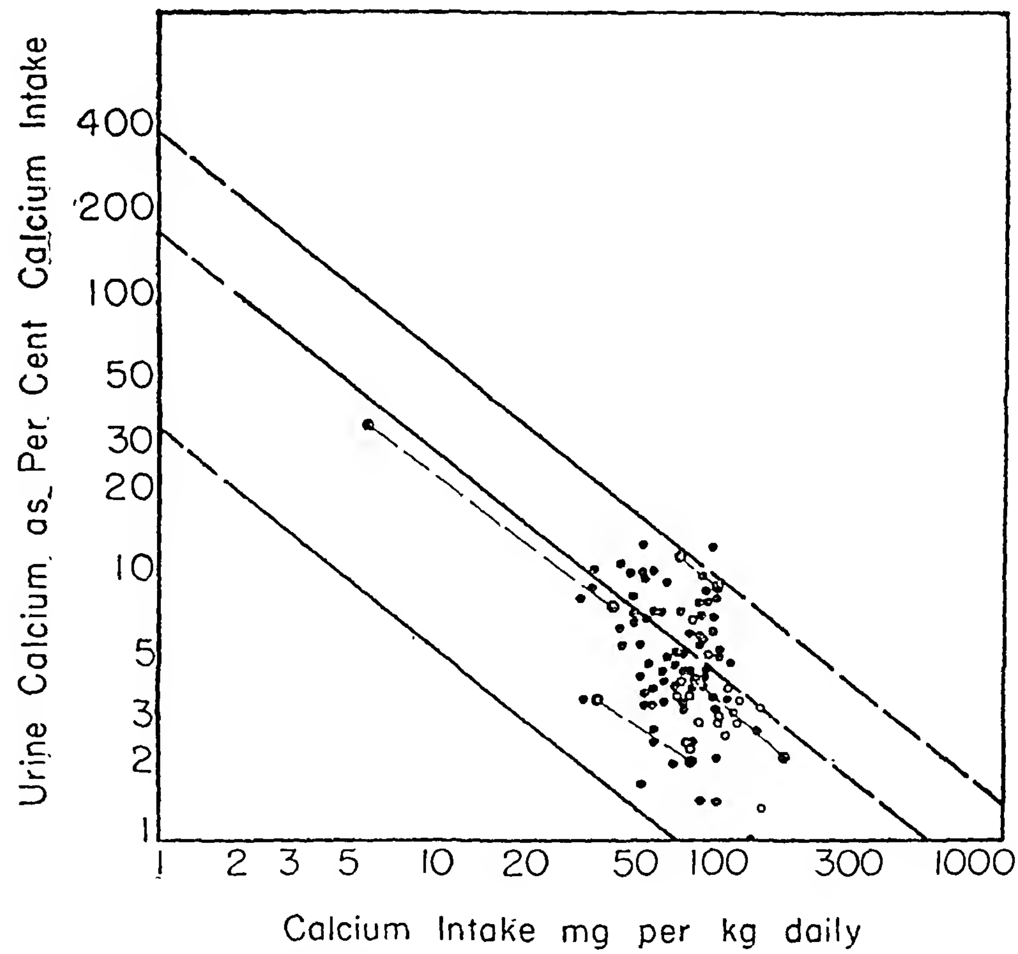

FIG. 4. URINARY CALCIUM VALUES FOR CHILDREN 1 TO 5 YEARS OLD

Solid lines represent mean, maximum, and minimum curves as in Figure 3. The dotted lines connect values obtained from one child at two or more levels of calcium intake. The hollow symbols show urinary calcium values of children 1 to 2 years old.

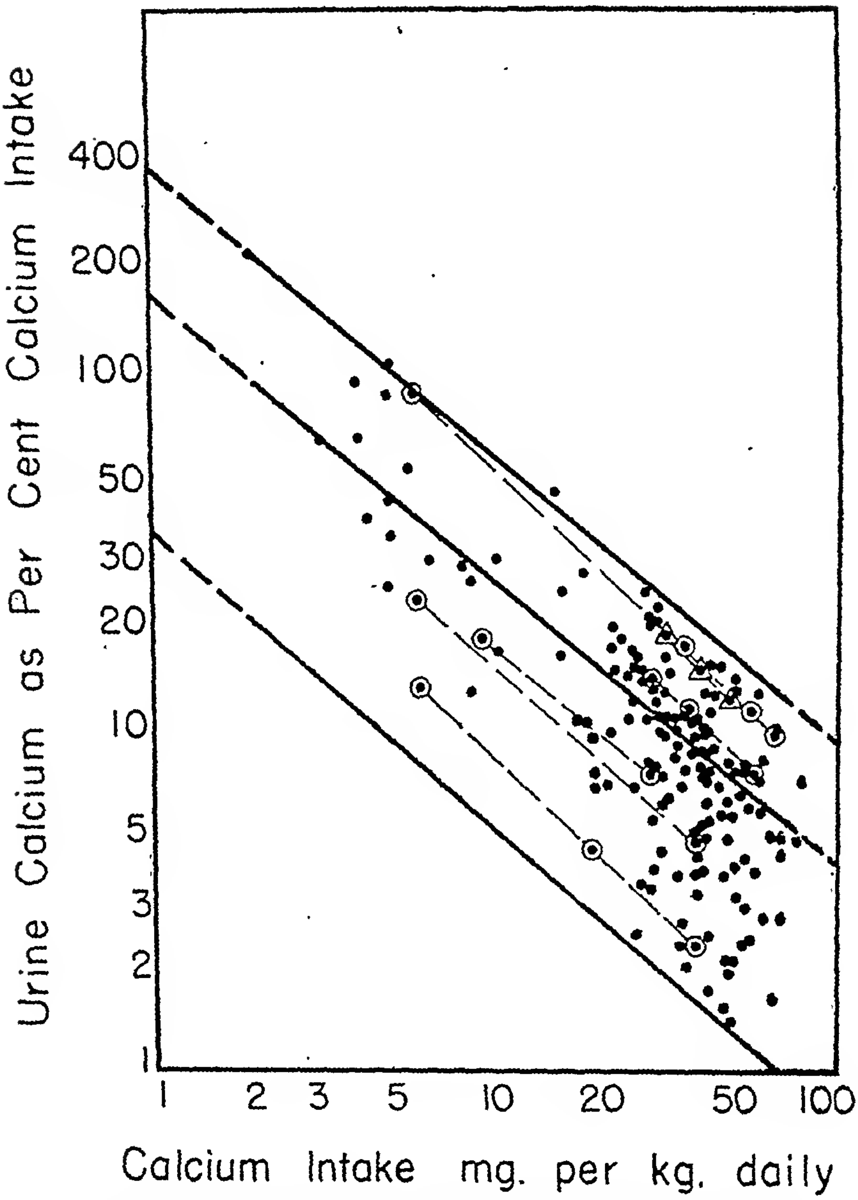

FIG. 5. URINARY CALCIUM VALUES FOR CHILDREN 10 TO 15 YEARS OLD
Solid and dotted lines as in Figure 4.

well within the normal range. The significance of this finding will be discussed later.

The relationships between calcium intake and urinary excretion set forth in Figures 3 to 6 were derived from data on all subjects, irrespective of calcium retention. In order to determine whether a total calcium excretion greater than the calcium intake would modify this relationship, the data for all subjects with negative calcium retentions were plotted as shown in Figure 7. It is obvious that the relationship expressed by the equations for mean, maximum, and minimum excretion and denoted by the lines in Figure 7 is equally valid for subjects in positive and negative calcium balance. In passing, one may note that a calcium intake of 20 mgm. per kgm. seems to be the critical level for negative calcium retentions. Irrespective of age, very few subjects lost calcium from the body when the calcium intake was greater than 20 mgm. per kgm.

A log-log chart of the maximum, mean, and minimum values for urinary calcium has proved

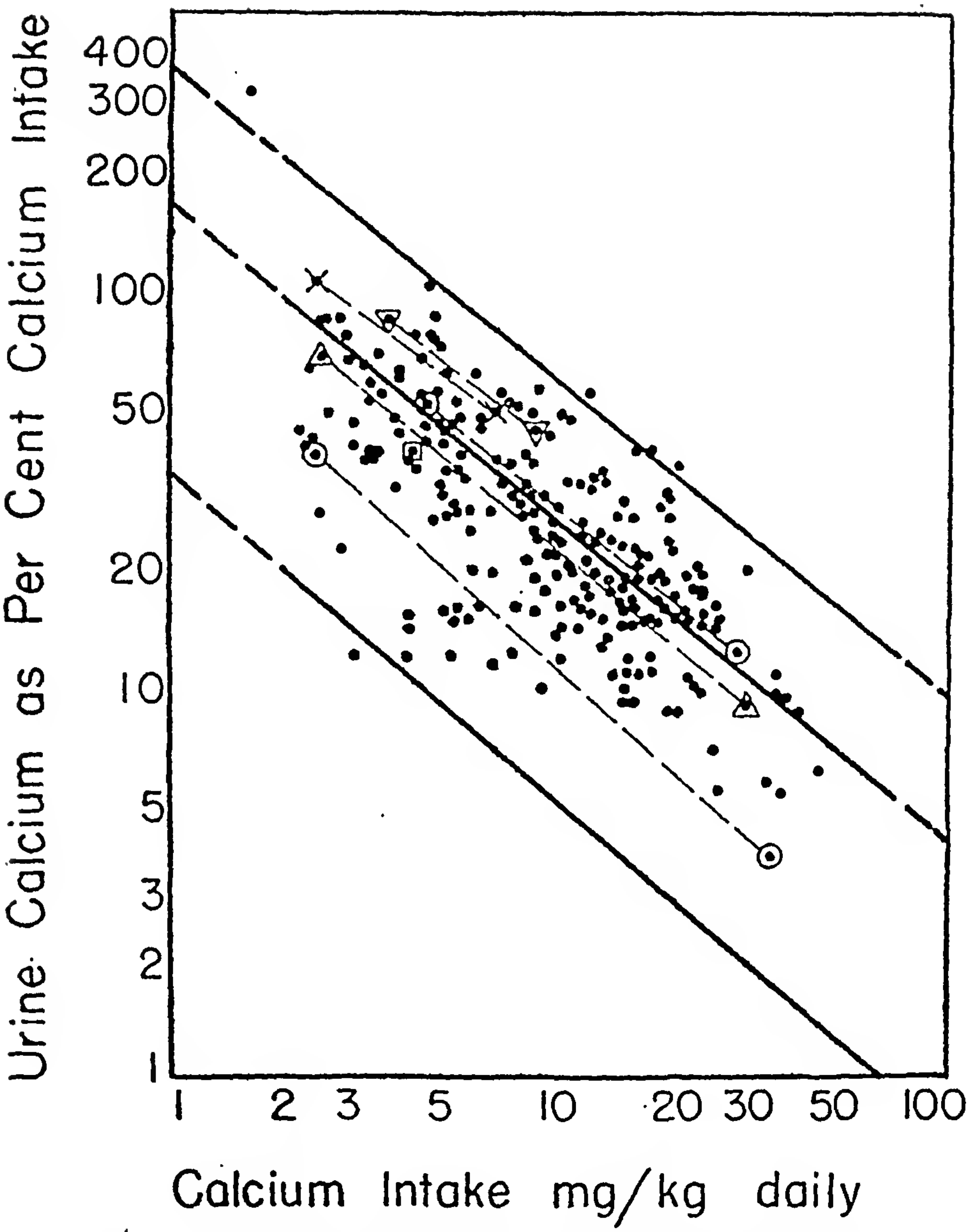

FIG. 6. URINARY CALCIUM VALUES FOR ADULTS 20 TO 80 YEARS OLD
Solid and dotted lines as in Figure 4.

useful in checking the normality of urinary calcium excretion in hospital patients. Three values only are needed for the determination: the daily calcium intake, 24-hour urinary calcium, and the weight of the person. If the milk intake is known, the dietary calcium may be estimated with sufficient accuracy for the large percentage of cases wherein the true values will lie close to the mean. For example, a 20-kgm. child drinks a pint of milk daily and excretes 100 mgm. of calcium in the urine. The true calcium intake would probably lie between 700 and 900 mgm. daily, or 35 to 45 mgm. per kgm. The urine calcium is between 14 and 11 per cent of these intakes. The ranges of these two values outline a square lying above the mean but well within the normal range. Had the urinary calcium been 200 mgm. daily, the values of 22 to 28 per cent of intake would indicate a urine calcium above the normal range, and an accurate study would be indicated.

Urinary calcium in infancy

The urinary excretion of calcium during the first year of life has been considered separately from that of the older subjects. Data were available from 980 3-day studies of 95 normal male infants fed cow's milk and ranging in age from 3 to 52 weeks (46, 48) and for 31 studies of in-

fants under 26 weeks of age given human milk exclusively (49–52).

Each infant given cow's milk received 350 I.U. of vitamin D daily and retained ample amounts of calcium. The daily urine calcium excretion was relatively small, from 1 to 82 mgm. for the infants under 6 months of age and from 3 to 86 mgm. for those over 6 months. The variability of urinary calcium was much greater among the group of infants and for the same infant during the first year of life than was observed with the older children.

The $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ of infants fed cow's milk was compared with the intake per kgm. The mean values obtained for each week of age are plotted in Figure 8. The mean values are consistently below the mean noted for the older subjects, but well within the normal range. The values for the youngest infants are lowest and the mean $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ tends to approach the mean curve with increasing age and decreasing per kilogram intake of the infants.

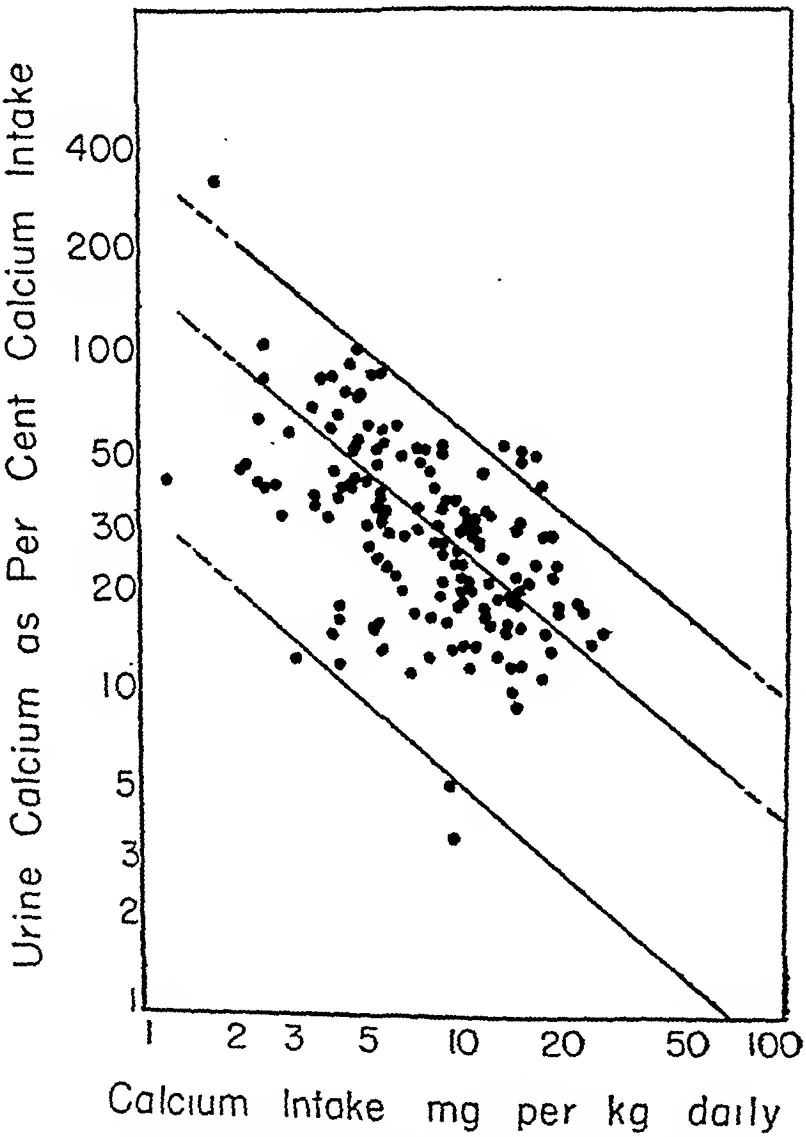

FIG. 7. URINARY CALCIUM VALUES FOR SUBJECTS IN NEGATIVE CALCIUM BALANCE

Solid lines represent mean, maximum, and minimum curves as in Figure 3. The data include 164 studies from subjects 4–76 years old.

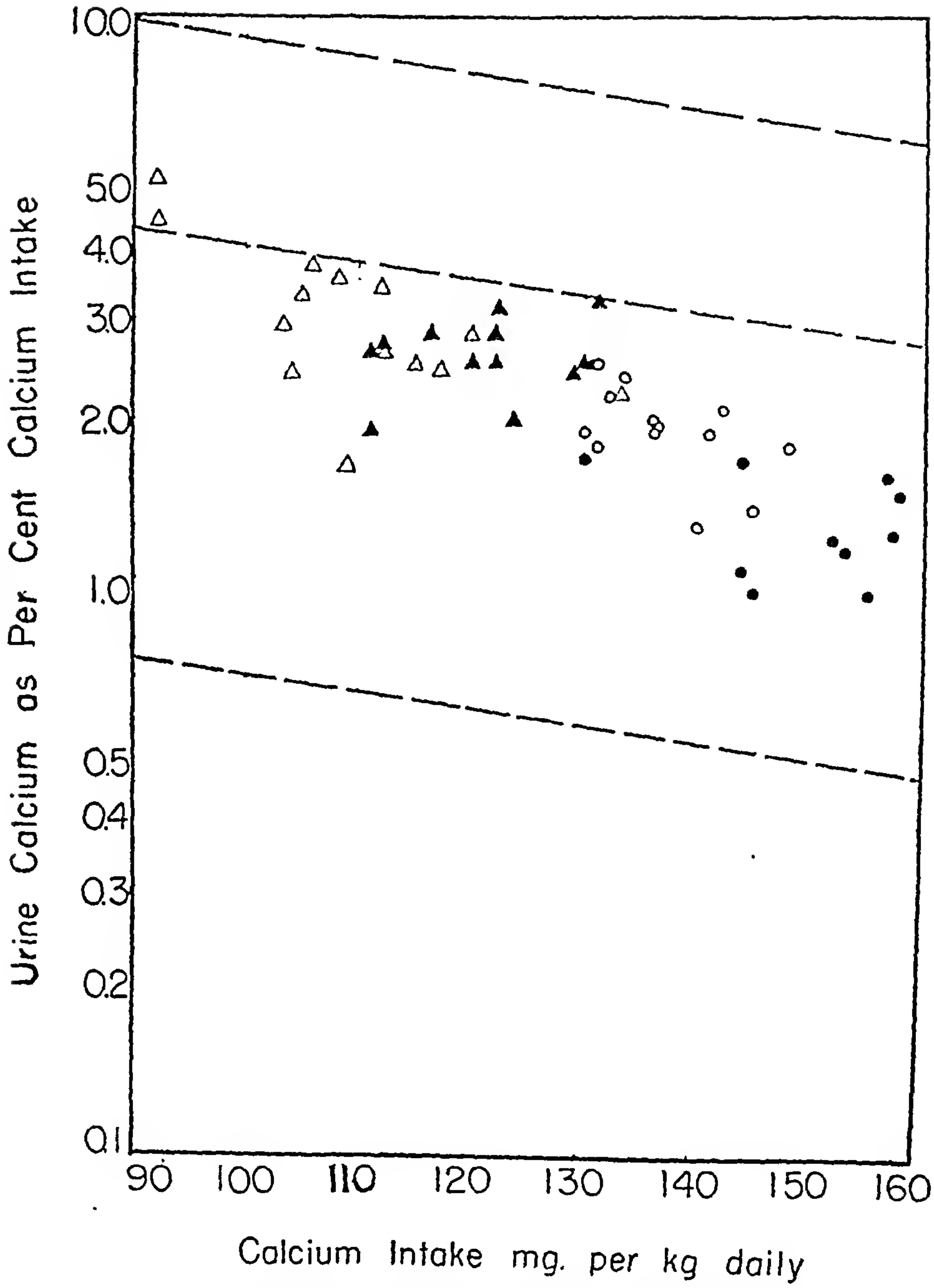

FIG. 8. URINARY CALCIUM VALUES FOR INFANTS FED COW'S MILK (47, 48)

Note that in this figure only the ordinate is on a logarithmic scale. Mean, maximum and minimum curves are extrapolated from those of Figure 3. Values are averaged for each week of age. Solid circles show average values for infants under 3 months old; open circles, those 3 to 6 months old; solid triangles, infants 6 to 9 months old; and open triangles, infants 9 to 12 months of age.

A similar study of the urinary calcium excretion of breast-fed infants whose daily calcium intakes varied from 44 to 125 mgm. per kgm., shows values comparable to those of the infants fed cow's milk. No consistent differences were observed between the values for babies under 3 months and those 3 to 6 months of age (Figure 9).

It thus appears that all normal infants, whether the per kgm. calcium intake be high or low, will excrete an amount of calcium in the urine that is within the range of normal values for older subjects, but below the mean for the latter group. It appears that the decrease in mean excretion is greatest during the first 6 months of life and tends to approach the normal during later infancy.

From the findings in Figure 4, in which the values for $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ for children 1 to 2 years of age are still below the mean, it seems probable that the mean level of excretion characteristic for older subjects is not achieved until the child is about 2 years old.

The decrease in urinary calcium observed during infancy has two possible causes. Although individual babies, over a period of several months, tend to maintain a fairly constant level of excretion in relation to the mean, it may be that the endocrine glands, the secretions of which we believe make up the "endogenous factor," function less efficiently in the young infant than in the older child. As an example, hypoparathyroid tetany of the new-born is occasionally observed and appears to be always a transitory disturbance.

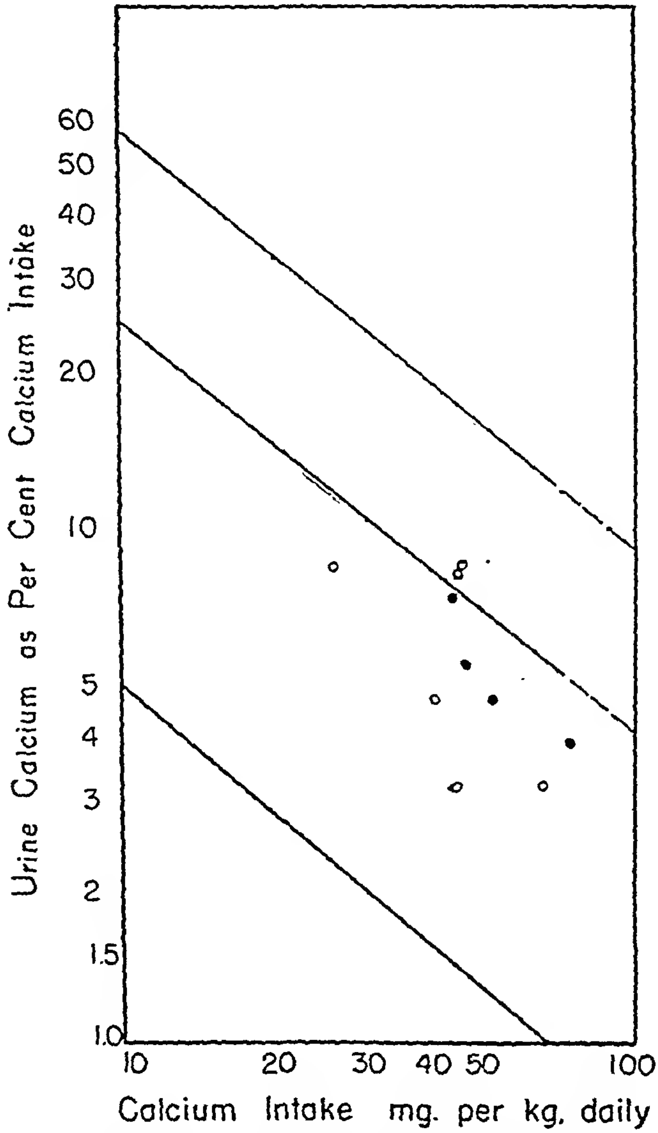

FIG. 9. URINARY CALCIUM VALUES FOR INFANTS FED HUMAN MILK (47, 49–52)

Lines as in Figure 8. The data have been averaged for each week of age. Hollow symbols represent values for infants under 3 months of age; solid symbols, infants 3 to 6 months old.

Also, the relatively marked calcium undersaturation of the skeleton in early infancy may have the same effect on decreasing urinary calcium as does severe calcium undernutrition in older subjects. It appears highly probable that both factors are active in determining the quantity of calcium excreted in the urine of infants.

Undernutrition and overnutrition

During recovery from severe undernutrition, especially calcium undernutrition, the relative unsaturation of bone may be sufficient to cause an unusually large deposition of calcium in bone and result in lowered urinary excretion of calcium. The fact that urine calcium values of infants are below the mean value for older children and adults lends support to this possibility.

Several studies of undernourished children (41, 53) and infants (54) are available for analysis. Figure 10 shows changes in $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ occurring as the children gained weight. For the children most seriously undernourished (30 to 45 per cent underweight), urine calcium values were

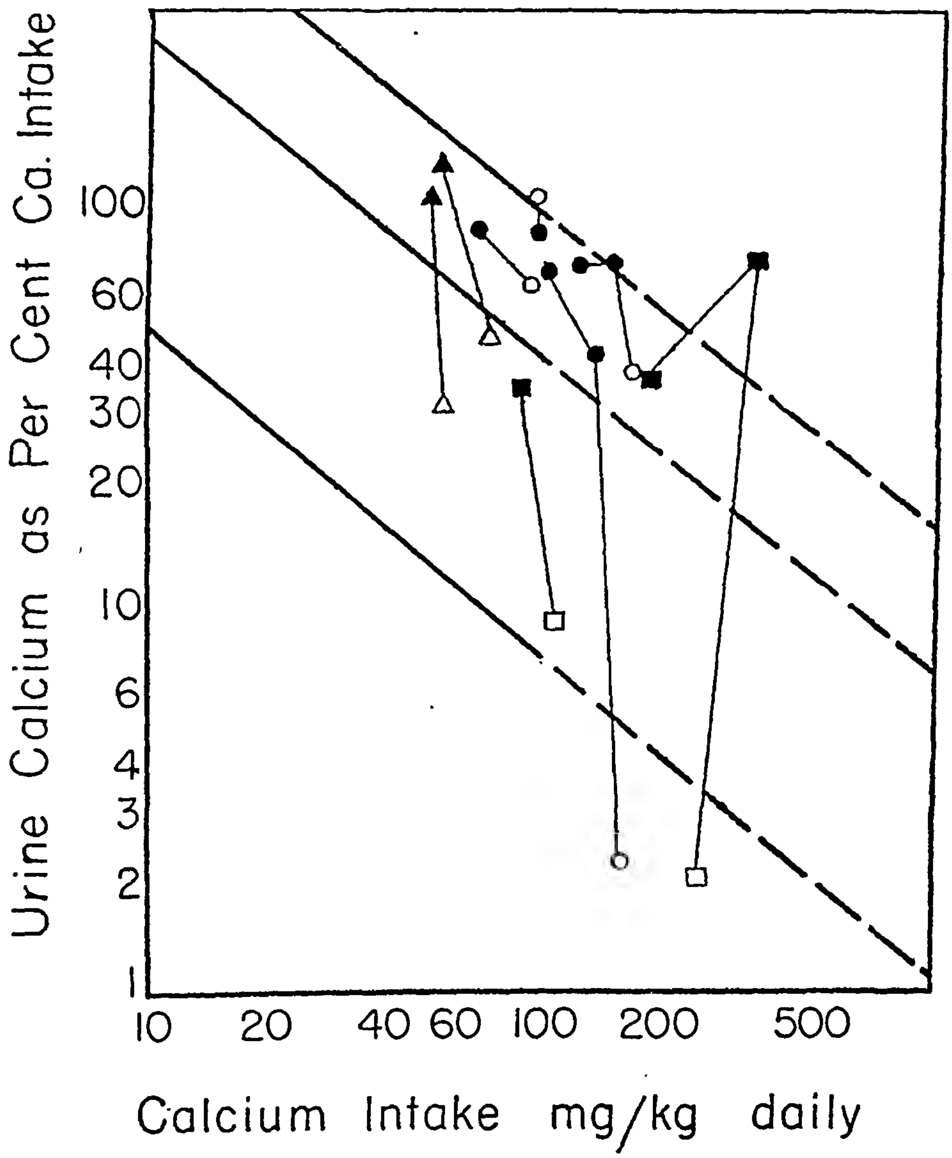

FIG. 10. URINARY CALCIUM VALUES FOR CHILDREN DURING RECOVERY FROM SEVERE MALNUTRITION

The hollow symbols represent the first observation, when the child was most underweight. In most of the cases of severe undernutrition the urinary calcium increased with the gain in weight.

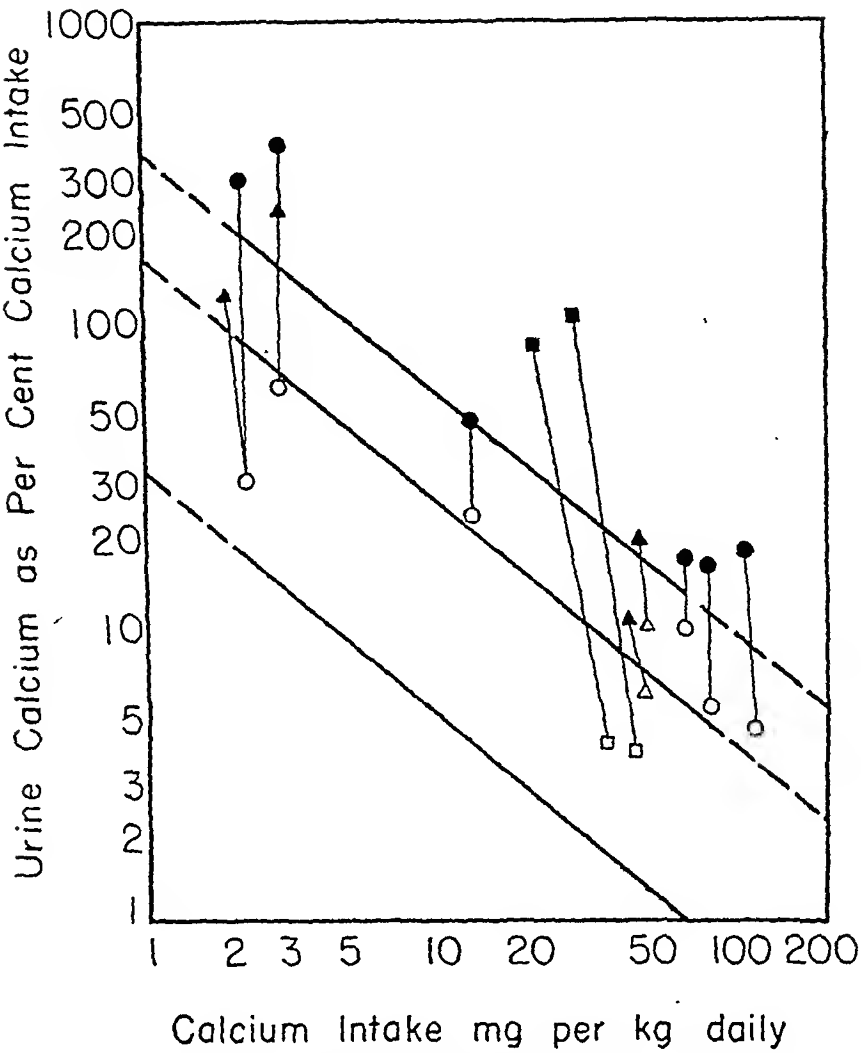

FIG. 11. THE EFFECT OF INCREASING METABOLIC ACIDITY ON EXCRETION OF URINARY CALCIUM

The background lines represent the normal maximum, mean, and minimum $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ in relation to calcium intake in mgm. per kgm. Hollow symbols represent control studies with a neutral ash diet; solid symbols, findings after increasing the metabolic acidity by ketogenic diet (■), NH_4Cl by mouth (●), or by ingestion of an acid-ash diet (▲). It is obvious that the first two methods of increasing metabolic acidity can result in increasing urinary excretion of calcium to above normal limits.

uniformly low and increased sharply as the child's weight approached normal. Some children less severely undernourished showed lowered urinary calcium values during the period of undernutrition, but several of this group excreted as much calcium in the urine during the period of undernutrition as when normal weight was attained.

The effect of calcium undernutrition *per se* can be illustrated by a study of a woman of 34 with severe osteoporosis (55). In this subject the presence of a low-grade steatorrhea for over 10 years, although symptomless, had led to a severe osteomalacia even though the milk intake had been adequate. The urinary calcium excretion before treatment was 16 mgm. daily or 1.2 per cent of the calcium intake of 40 mgm. per kgm. This value is minimum normal. She was not retaining calcium. Treatment consisted of cod

liver oil, bile salts, and pancreatin in conjunction with a diet high in calcium and phosphorus and low in fat content. After 4 months on this regimen, the patient showed dramatic improvement; she gained weight and the osteoporosis was very nearly healed. The calcium retention at this time approximated 0.5 gram daily. However, the urinary calcium excretion was only 9 to 11 mgm. daily, or 0.5 to 0.7 of the intake of 36 mgm. per kgm., values well below the normal minimum. Practically all the absorbed calcium was being retained in the bones, and very little remained to be excreted in the urine. Similar low values for urine calcium may be observed during recovery from hyperparathyroidism.

In obesity, there seems no reason to assume abnormality of calcium excretion, unless the obesity is accompanied by endocrine disturbance. As it seems probable that the relationship observed between weight of the subject and urinary calcium is due primarily to a relationship between skeletal weight and urinary calcium, the urinary calcium may be expected to be low. Therefore, the use of theoretical rather than actual weight of the obese individual seems preferable in calculating normality of urinary calcium. For example, a boy of 11 during reduction of weight from 124.5 to 99 kgm. excreted 8.5 to 9.3 per cent of his calcium intake of 9 to 14 mgm. per kgm. actual weight, or 26 to 33 mgm. per kgm. theoretical weight. These values are within normal limits for both intake levels, approach the mean value for theoretical per kgm. intake, and are well below the mean value for actual per kgm. intake.

THE INFLUENCE OF DIETARY FACTORS OTHER THAN CALCIUM INTAKE

In addition to the major factors affecting urinary calcium, namely, calcium intake, amount of bone measured by body weight, and endogenous factor, other factors exert lesser effects, which though not great in themselves, must be considered in planning dietary regimens whenever urinary calcium is to be studied. Of these other factors, the increase of acid metabolites in the body, the quantity of protein ingested, the calcium-phosphorus intake ratio, dietary roughage, vitamin D, and the possible effects of intake of other salts have all been discussed as factors affecting urinary calcium (56–73). The relative importance of these factors can now be evaluated.

The increase of acid ions in the body may be accomplished by feeding of mineral acids or acid salts such as ammonium chloride, by producing ketosis through the use of a high fat diet, or by the use of a diet giving an acid ash. Figure 11 shows the effect of these factors on urinary calcium. All data were taken from the literature (27, 56–58), and in every study each subject served as his own control. The calcium intake was nearly constant in all studies except one (27) in which the experimental diet contained less calcium than the control. Ingestion of ketogenic diet (fatty acid: glucose = 2.6) by children resulted in the most marked increase in urinary calcium. Values for $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ were increased far above the normal maximum. Ammonium chloride increased the urine calcium excretion of children (27) and adults (56) above the normal maximum. Ingestion of an acid-ash diet by adults (56), although less effective than NH_4Cl, did increase the urine calcium from values near the mean to values near the normal maximum level of excretion. The importance of ingestion of a neutral-ash diet when urinary calcium excretion is studied is thus emphasized.

An additional factor known to influence the quantity of urinary calcium is the ratio of calcium to phosphorus in the diet. The data shown in Figure 12 taken from studies made in this laboratory (46, 61) illustrate the effect of changing the calcium to phosphorus ratio without altering the total calcium content of the diet. The high calcium to phosphorus intake ratios were attained by giving young adults a constant basal diet and providing the dietary calcium as calcium phosphate for the control period and as calcium gluconate or lactate for the experimental periods. The data for the lower calcium intake levels were obtained by giving children a low calcium diet and decreasing the calcium to phosphorus ratio by the addition of sodium glycerophosphate to the diet. The results show consistent increase in $\frac{\text{urine Ca} \times 100}{\text{intake Ca}}$ with increasing calcium to phosphorus intake ratio and constant calcium intake. In some cases, the increases in urinary calcium

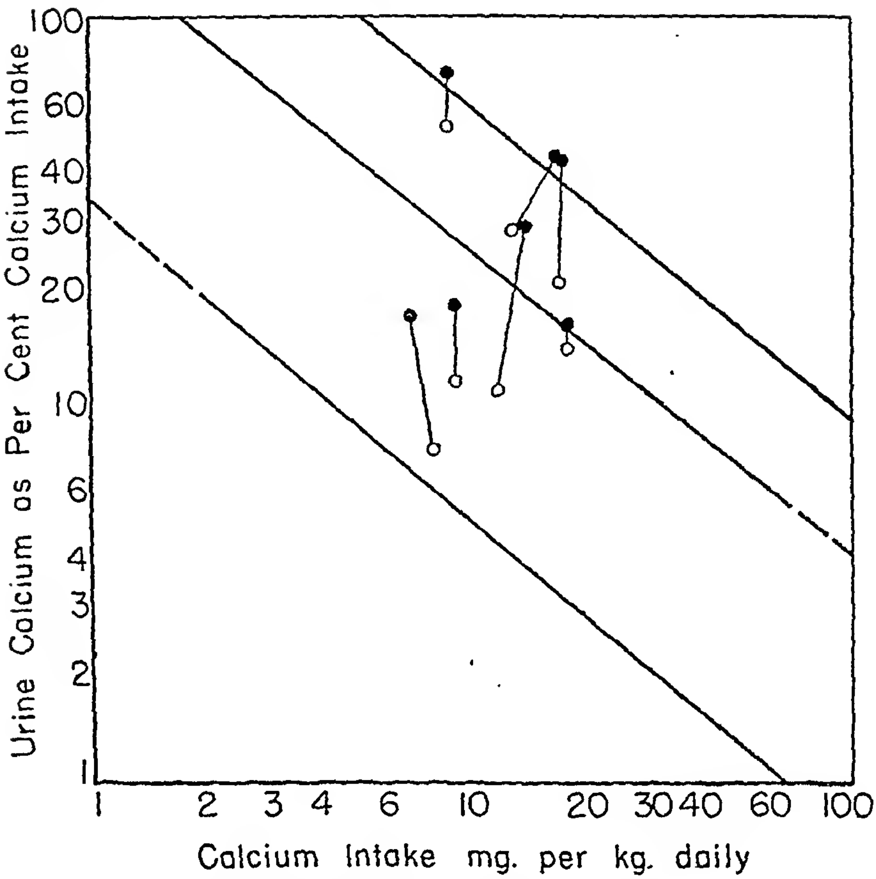

FIG. 12. THE EFFECT OF INCREASING THE CALCIUM TO PHOSPHORUS INTAKE RATIO ON THE EXCRETION OF URINARY CALCIUM

The hollow symbols represent the control studies and always indicate the lower calcium to phosphorus ratio of each pair. Increasing the calcium to phosphorus intake ratio results in variable increases in the per cent of intake excreted in the urine.

were sufficient to bring the values above the normal range.

In a similar study of an infant (62), the dietary calcium to phosphorus ratio was changed from 1.3 to 2.8 and finally to 5.1 by changing the diet from a milk formula to a soybean mixture containing $CaCO_3$. The daily urinary calcium values with the 3 diets were 23, 86, and 286 mgm. respectively.

Another factor affecting urinary calcium is the quantity of dietary protein. The significance of several studies on this subject is obscured by the fact that concurrent changes occurred in acid-base and calcium-phosphorus ratios of the diet. A few studies are available, however, in which these factors were controlled [Pittman and Kunerth (18, 30), McCance *et al.* (59), and Hawkes *et al.* (60)], and wherein each subject served as his control. In these studies and in a study from our own laboratory (46), the diets were controlled by the addition of minerals where necessary, so that the calcium and phosphorus intakes and calcium to phosphorus ratios were held very nearly constant. Also acid-base ratios varied only slightly between the low and high protein diets in all the studies except that of McCance (59), in which no mention is made of possible changes in acidity of the diet on addition of protein. The changes in urinary calcium values obtained in these studies are shown in Figure 13. The figures for high calcium intakes were obtained in pre-school children (60) with relatively minor changes (from 3 to 4 mgm. per kgm. per day) in dietary protein. It appears that increase in dietary protein without

notable concurrent increase in acidity of ash of the diet or change in dietary calcium to phosphorus ratio causes a small but consistent increase in urinary calcium. However, the amount of increase was not sufficient to bring the urine calcium outside the normal limits in any of the studies reported.

The question of the mechanical effect of the extra bulk of the cellulose from vegetables on the absorption and excretion of calcium has been studied by several investigators. Particular interest is centered on such vegetables as also contain oxalate. The results of vegetable feeding are somewhat conflicting and the changes in urine calcium are rarely marked. Spinach given to adults (63) decreased both absorption and urinary excretion of calcium. With children, spinach feeding was without significant effect on urine calcium (5), while studies of 4 infants fed small amounts of spinach gave variable results (64). Figure 14 records mean values for urine calcium of infants, children, and adults given diets of low and high vegetable content. In general, the feeding of increased amounts of vegetables appeared to decrease the urinary calcium, particularly when the chief source of intake calcium was vegetables (22, 31, 32, 38, 63), but the effect of the roughage was minor and inconsistent.

The well-known effect of vitamin D in increasing the retention of calcium in the body is usually considered to be the result of increased absorption of calcium from the intestine. Vitamin D given to rachitic infants increases both the absorption and urinary excretion of calcium. However, in amounts of about 400 I.U. daily, vitamin D has small and inconsistent effects on urinary calcium

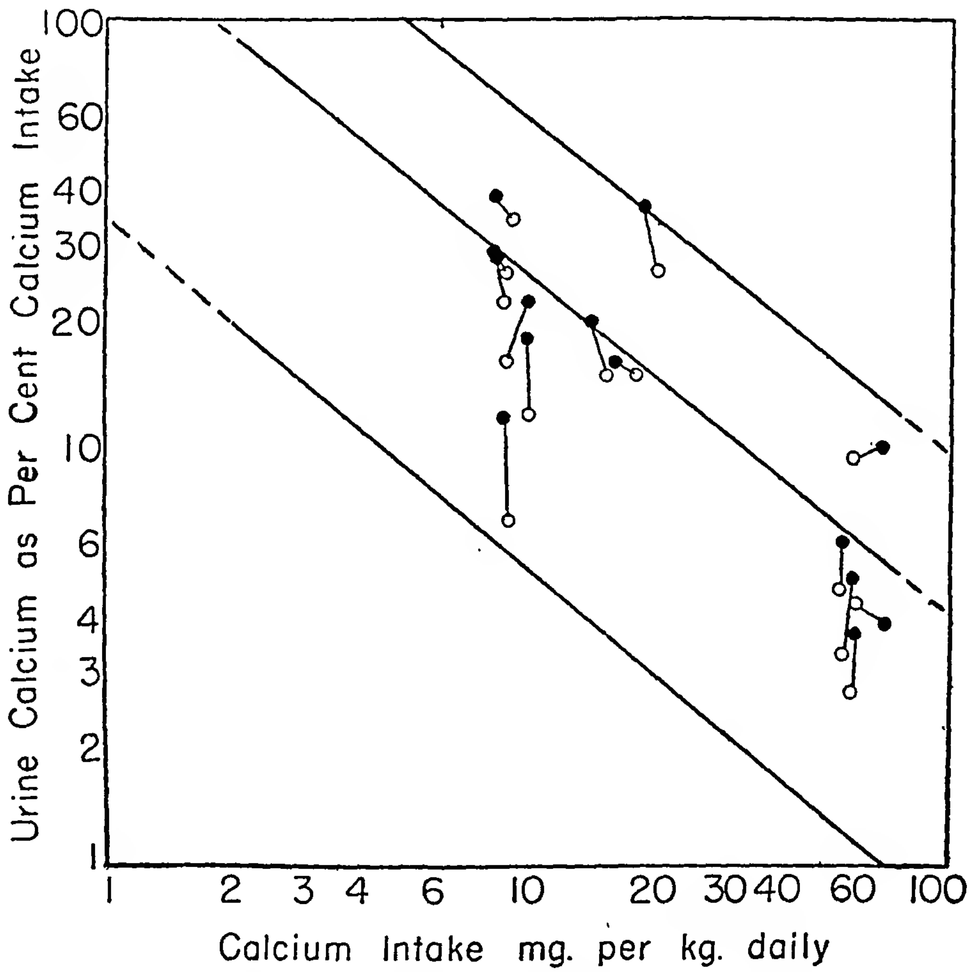

Fig. 13. The Effect of Increasing Protein Intake Without Increasing the Acidity of Ash of the Diet or the Calcium to Phosphorus Intake Ratio

Background lines and symbols are the same as in Figure 11. Increasing the protein intake results in small increases in urinary calcium excretion which do not exceed the normal limits.

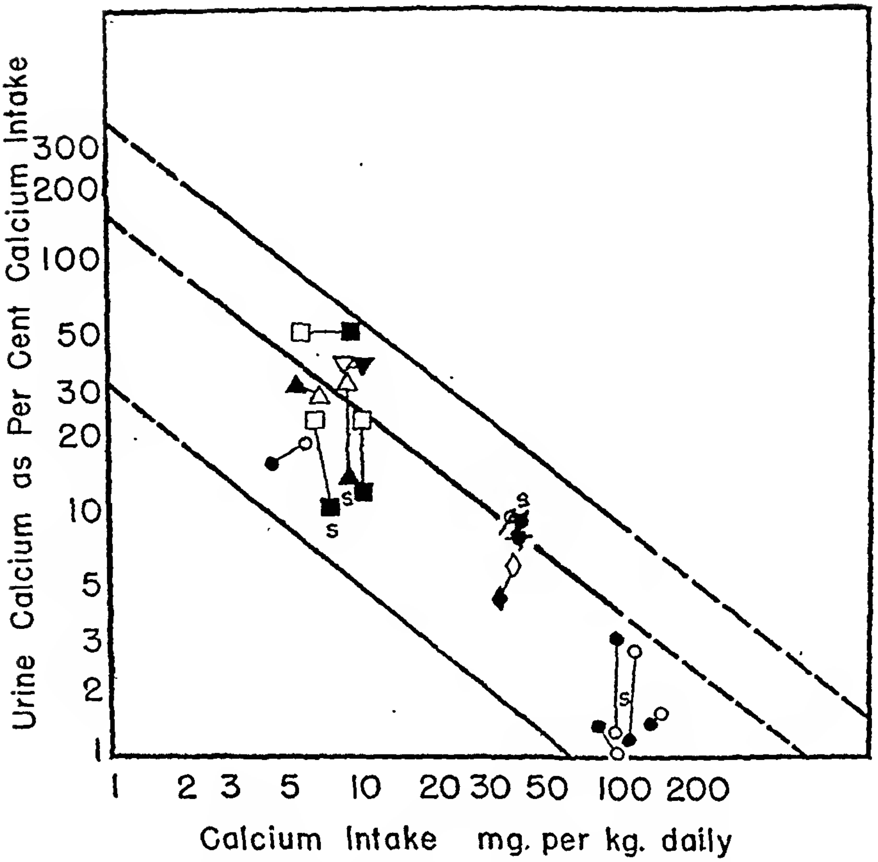

FIG. 14. THE EFFECT OF INCREASED INTAKE OF VEGETABLES ON EXCRETION OF URINARY CALCIUM

Solid symbols represent the higher roughage intake, hollow symbols represent control studies. S indicates that the vegetable studied was spinach.

of healthy children. In one study of 8 children, a consistent slight increase in average urinary calcium was noted (9); in another, no change was observed (66). Data from studies made in this laboratory on boys given 340 I.U. vitamin D as cod liver oil daily at two levels of calcium intake are presented in Table VIII. The administration of vitamin D appeared to increase urinary calcium slightly in 11 of 15 subjects when the calcium intake was moderate. With more ample calcium intake, the changes in urinary calcium were very small and not consistent. Similarly for 8 adolescent girls receiving ample calcium intake (Table VIII), 300 to 400 I.U. of vitamin D daily increased calcium absorption but caused no change in the urinary excretion of calcium. In adults the effect of moderate doses of vitamin D on urinary calcium is negligible (13, 16, 23, 67).

In hypervitaminosis D, the urinary calcium is usually greatly increased above the normal (68).

TABLE VIII

Influence of vitamin D intake on urinary excretion of calcium by children 1–15 years old

Age	No. subj.	Approx. intake	Vitamin D per day: 0 — Urine Mean	Vitamin D per day: 0 — Urine Range	Vitamin D per day: 300–400 I.U. — Urine Mean	Vitamin D per day: 300–400 I.U. — Urine Range
years		*grams per day*	*mgm. per day*		*mgm. per day*	
Boys						
1–3	2	0.750	29	13–45	43	36–50
1–3	4	1.350	36	27–55	38	25–52
4–7	7	0.750	65	33–128	80	24–142
4–7	6	1.450	94	76–165	79	46–181
8–12	6	0.850	82	40–122	174	114–240
8–12	5	1.650	186	83–279	112	60–159
Girls						
11–15	8	1.270	118	26–224	110	25–210

Doses of vitamin D much larger than are physiological, although not necessarily large enough to produce hypervitaminosis, are also reported to increase urinary calcium (67, 69).

Increases in urinary calcium have been noted after ingestion of large amounts of urea (70), magnesium citrate (3), and magnesium lactate (72) by adults. Lactose caused a consistent decrease in urinary calcium of 5 little boys (71). Small increases in urinary calcium were noted after addition of sodium or potassium chloride to the diet of infants studied in this laboratory (61) and in adults (73). Potassium citrate consistently lowered urinary calcium excretion of 8 preschool children (74).

Because dietary factors such as acid-base balance, calcium to phosphorus ratio, and other factors discussed above modify somewhat the level of urinary calcium excretion characteristic for the individual, it is important that they be controlled in any study of the effect of other factors, particularly when small groups of subjects are used.

SUMMARY AND CONCLUSIONS

Through the study of the urinary calcium excretion of 606 normal persons, from 1 to 80 years of age, under standard dietary regulation, the normal range of urinary excretion of calcium has been established and a new tool provided for the study of calcium metabolism.

1. The quantity of urine calcium is dependent on an endogenous factor or factors, presumably endocrine, and on calcium intake per unit of weight. Age and sex are not factors except as they affect skeletal weight. It is thus possible to compare data from subjects of all ages and with varying dietary intakes.

2. Urinary calcium expressed as per cent of calcium intake varies inversely with the intake per kgm. and is an exponential function of the latter. Values for mean, minimum, and maximum normal urinary calcium may be expressed by specific equations.

3. The urinary calcium excretion of subjects below 2 years of age is within the normal range but below the mean value for older children and adults.

4. Dietary factors other than calcium intake have relatively minor effects on urine calcium, with the exception of ingested acids, ketogenic diets, or diets with a high calcium to phosphorus ratio, all of which increase urinary calcium, often above maximum normal limits.

5. The ability to demarcate the normal range of urinary calcium with a considerable degree of accuracy should prove a useful aid in further studies of calcium metabolism, as well as an aid in diagnosis of disease entities associated with alteration of urinary excretion of calcium.

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BIBLIOGRAPHY

1. Bauer, W., Albright, F., and Aub, J. C., Studies of calcium and phosphorus metabolism. II. The calcium excretion of normal individuals on a low calcium diet, also data on a case of pregnancy. J. Clin. Invest., 1929, 7, 75.
2. Blatherwick, N. R., and Long, M. L., The utilization of calcium and phosphorus of vegetables by man. J. Biol. Chem., 1922, 52, 125.
3. Bogert, L. J., and McKittrick, E. J., Studies in inorganic metabolism. I. Interrelations between calcium and magnesium metabolism. J. Biol. Chem., 1922, 54, 363.
4. Bogert, L. J., and Kirkpatrick, E. E., Studies in inorganic metabolism. II. The effects of acid-forming and base-forming diets upon calcium metabolism. J. Biol. Chem., 1922, 54, 375.
5. Bonner, P., Hummel, F. C., Bates, M. F., Horton, J., Hunscher, H. A., and Macy, I. G., The influence of a daily serving of spinach or its equivalent in oxalic acid upon the mineral utilization of children. J. Pediat., 1938, 12, 188.
6. Breiter, H., Mills, R., Dwight, J., McKey, B., Armstrong, W., and Outhouse, J., The utilization of the calcium of milk by adults. J. Nutrition, 1941, 21, 351.
7. Burton, Helen B., The influence of cereals upon the retention of calcium and phosphorus in children and adults. J. Biol. Chem., 1930, 85, 405.
8. Chaney, M. S., and Blunt, K., The effect of orange juice on the calcium, phosphorus, magnesium, and nitrogen retention and urinary organic acids of growing children. J. Biol. Chem., 1925, 66, 829.
9. Daniels, A. L., Hutton, M. K., Knott, E., Everson, G., and Wright, O., Relation of ingestion of milk to calcium metabolism in children. Am. J. Dis. Child., 1934, 47, 499.
10. Davis, N. J., Calcium, phosphorus, and nitrogen retention of children. Effects of acid-forming and

base-forming diets. Am. J. Dis. Child., 1935, 49, 611.

11. Discli, Anne C., The effect of an acid ash diet upon the calcium and phosphorus excretion of normal adults and kidney stone patients. Thesis, State Univ. Iowa, 1941.

12. Farquharson, R. F., Salter, W. T., and Aub, J. C., Studies of calcium and phosphorus metabolism. XIII. The effect of ingestion of phosphates on the excretion of calcium. J. Clin. Invest., 1931, 10, 251.

13. Hart, M. C., Tourtellotte, D., and Heyl, F. W., The effect of irradiation and cod liver oil on the calcium balance in the adult human. J. Biol. Chem., 1928, 76, 143.

14. Herbst, O., Beitrage zur Physiologie des Stoffwechsels in Knaben-alter mit besonderer Berucksichtigung einiger Mineralstoffe. Annales Paediatrici, 1912, 76, 40.

15. Herbst, O., Calcium and Phosphor beim Wachstum am Ende der Kindheit. Ztschr. f. Kinderh., 1913, 7, 161.

16. Hunscher, H. A., Donelson, E., Erickson, B. N., and Macy, I. G., Results of the ingestion of cod liver oil and yeast on calcium and phosphorus metabolism of women. J. Nutrition, 1934, 8, 341.

17. Johnston, J. A., Factors influencing retention of nitrogen and calcium in period of growth. IV. Effect of estrogen. Am. J. Dis. Child., 1941, 62, 708.

18. Kunerth, B. L., and Pittman, M. S., A long-time study of nitrogen, calcium, and phosphorus metabolism on a low-protein diet. J. Nutrition, 1939, 17, 161.

19. Leverton, R. M., and Marsh, A. G., One hundred studies of the calcium, phosphorus, iron, and nitrogen metabolism and requirement of young women. Univ. Nebr. Agric. Expt. Sta., Research Bull., 1942, 125.

20. Mallon, M. G., Jordan, R., and Johnson, M., A note on the calcium retention on a high and low fat diet. J. Biol. Chem., 1930, 88, 163.

21. Mallon, M. G., Johnson, L. M., and Darby, C. R., A study of the calcium retention on a diet containing American cheddar cheese. J. Nutrition, 1932, 5, 121.

22. Mallon, M. G., Johnson, L. M., and Darby, C. R., The calcium retention on a diet containing leaf lettuce. J. Nutrition, 1933, 6, 303.

23. McCance, R. A., and Widdowson, E. M., Mineral metabolism of healthy adults on white and brown bread dietaries. J. Physiol., 1942, 101, 44.

24. McCance, R. A., Widdowson, E. M., and Lehmann, H., The effect of protein intake on the absorption of calcium and magnesium. Biochem. J., 1942, 36, 686.

25. McClellan, W. S., Rupp, V. R., and Toscani, V., Clinical calorimetry. XLVI. Prolonged meat diets with a study of the metabolism of nitrogen, calcium, and phosphorus. J. Biol. Chem., 1930, 87, 669.

26. Unpublished data from the study reported by McKay, H., Patton, M. B., Ohlson, M. A., Pittman, M. S., Leverton, R. M., Marsh, A. G., Stearns, G., and Cox, G., Calcium, phosphorus, and nitrogen metabolism of young college women. J. Nutrition, 1942, 24, 367.

27. Morris, N., and MacRae, O., Metabolic reactions to acidosis produced by ammonium chloride. Arch. Dis. Childhood, 1930, 5, 207.

28. Owen, E. C., Irving, J. T., and Lyall, A., The calcium requirements of older male subjects with special reference to the genesis of senile osteoporosis. Acta Med. Scandinav., 1940, 103, 235.

29. Pierce, H. B., Daggs, R. G., Meservey, A. B., and Simcox, W. J., The retention of calcium and phosphorus by pre-school children. J. Nutrition, 1940, 19, 401.

30. Pittman, M. S., and Kunerth, B. L., A long-time study of nitrogen, calcium, and phosphorus metabolism on a medium-protein diet. J. Nutrition, 1939, 17, 175.

31. Rose, M. S., Experiments on the utilization of the calcium of carrots by man. J. Biol. Chem., 1920, 41, 349.

32. Rose, M. S., and MacLeod, G., Experiments on the utilization of the calcium of almonds by man. J. Biol. Chem., 1923, 57, 305.

33. Sahs, E. B., Dietary factors influencing urinary acidity and stone formation. Thesis, State Univ. Iowa, 1939.

34. Sherman, H. C., Gillette, L. H., and Pope, H. M., Monthly metabolism of nitrogen, phosphorus, and calcium in healthy women. J. Biol. Chem., 1918, 34, 373.

35. Sherman, H. C., Wheeler, L., and Yates, A. B., Experiments on the nutritive value of maize protein and on the phosphorus and calcium requirements of healthy women. J. Biol. Chem., 1918, 34, 383.

36. Sherman, H. C., Winters, J. C., and Phillips, V., Efficiency of oat protein in adult human nutrition. J. Biol. Chem., 1919, 39, 53.

37. Sherman, H. C., Calcium requirement of maintenance in man. J. Biol. Chem., 1920, 44, 21.

38. Sherman, H. C., and Hawley, E., Calcium and phosphorus metabolism in childhood. J. Biol. Chem., 1922, 53, 375.

39. Steggerda, F. R., and Mitchell, H. H., The calcium requirement of adult man and the utilization of the calcium in milk and in calcium gluconate. J. Nutrition, 1939, 17, 253.

40. Steggerda, F. R., and Mitchell, H. H., Further experiments on the calcium requirement of adult man and the utilization of calcium in milk. J. Nutrition, 1941, 21, 577.

41. Wang, C. C., Kaucher, M., and Frank, M., Metabolism of undernourished children. IV. Calcium metabolism. Am. J. Dis. Child., 1928, 35, 856.

42. Wang, C. C., Kern, R., and Kaucher, M., Minimum requirement of calcium and phosphorus in children. Am. J. Dis. Child., 1930, 39, 768.

43. Wang, C. C., Kaucher, M., and Wing, M., Metabolism of adolescent girls. IV. Mineral metabolism. Am. J. Dis. Child., 1936, 52, 41.
44. Willard, A. C., and Blunt, K., A comparison of evaporated with pasteurized milk as a source of calcium, phosphorus, and nitrogen. J. Biol. Chem., 1927, 75, 251.
45. McCrudden, F. H., The determination of calcium in the presence of magnesium and phosphates: the determination of calcium in urine. J. Biol. Chem., 1911–12, 10, 187.
46. Knapp, E. L., Studies on the urinary excretion of calcium. Thesis, State University of Iowa, 1943.
47. Unpublished data; Jeans, P. C., Stearns, G., and others.
48. Jeans, P. C., Stearns, G., McKinley, J. B., Goff, E. A., and Stinger, D., Factors possibly influencing the retention of calcium, phosphorus, and nitrogen by infants given whole milk feedings. I. The curding agent. J. Pediatrics, 1936, 8, 403.
49. Czerny, A. D., and Keller, A., Des Kindes Ernahrung, Ernahrungsstorungen, und Ernahrungstherapie, Vol. II. Leipzig, 1928.
50. Wang, C. C., Witt, D. B., and Felcher, A. R., A comparison of the metabolism of some mineral constituents of cow's milk and of breast milk in the same infant. Am. J. Dis. Child., 1924, 27, 352.
51. Lingberg, G., Über den Stoffwechsel des gesunden, naturlich ernährten Sauglings und dessen Beiinflussung durch Frauenmilchfett. Ztschr. f. Kinderh., 1917, 16, 90.
52. Muhl, Greta, Über den Stoffwechsel des gesunden naturlich ernährten Sauglings und dessen Beiinflussung durch Fettreduktion der Nahrung. Acta Paediat., 1923, 2, Suppl., 1–140.
53. a. Stearns, G., and Moore, D. L. R., Growth in height and weight, and retention of nitrogen, calcium, and phosphorus during recovery from severe malnutrition. Am. J. Dis. Child., 1931, 42, 774.
b. Stearns, G., Jeans, P. C., Catherwood, R., and McKinley, J. B., Diet in malnutrition and celiac disease. J. Pediat., 1941, 18, 12.
54. Utheim, K., Advanced chronic nutritional disturbances in infancy. Stud. from Dep't Pediat., Wash. U. School Med., 1922, 4, 1.
55. Miyakawa, G., and Stearns, G., Severe osteoporosis (or osteomalacia) associated with long-continued low-grade steatorrhea. J. Bone and Joint Surg., 1942, 24, 429.
56. Farquharson, R. F., Salter, W. T., Tibbetts, D. M., and Aub, J. C., Studies of calcium and phosphorus metabolism. XII. The effect of the ingestion of acid-producing substances. J. Clin. Invest., 1931, 10, 221.
57. Niermann, M., and Winter, M., Stoffwechseluntersuchungen bei Ernährung mit sauren Milchmischugen. Arch. f. Kinderh., 1930, 89, 290.
58. Nelson, M. V., Calcium and phosphorus metabolism of epileptic children receiving a ketogenic diet. Am. J. Dis. Child., 1928, 36, 716.
59. Lehmann, H., and Pollak, L., The effect of amino acids on phosphate transfer in muscle extract. Biochem. J., 1942, 36, 672.
60. Hawks, J. E., Bray, M. M., Wilde, M. O., and Dye, M., The interrelationship of calcium, phosphorus, and nitrogen in the metabolism of pre-school children. J. Nutrition, 1942, 24, 283.
61. Stearns, Genevieve, and others, unpublished data.
62. Stearns, G., Soybean flour in infant feeding. A study of the relation of the comparative intakes of nitrogen, calcium, and phosphorus on the excretion and retention of these elements by infants. Am. J. Dis. Child., 1933, 46, 7.
63. McLaughlin, Laura, Utilization of the calcium of spinach. J. Biol. Chem., 1927, 74, 455.
64. Stearns,, G., and Stinger, D., unpublished data.
65. Edelstein, E., Der Einfluss von Gemüsezulagen auf den Stickstoff und Mineralstoffwechsel des Kindes. Ztschr. f. Kinderh., 1932, 52, 483.
66. Weld, C. B., and Sykes, J. F., The effect of irradiated ergosterol on calcium and phosphorus retention in children. Trans. Roy. Soc. Canada, Sec. V, 1932, 81.
67. Bauer, W., Marble, A., and Claflin, D., Studies on the mode of action of irradiated ergosterol. I. Its effect on the calcium, phosphorus, and nitrogen metabolism of normal individuals. J. Clin. Invest., 1932, 11, 1.
68. Harris, L. J., The mode of action of vitamin D. The "parathyroid" theory: clinical hypervitaminosis. Lancet, 1932, 1, 1031.
69. Rose, E., Perloff, W. H., and Sundermann, F. W., The effect of low calcium diet and calciferol on calcium and phosphorus metabolism: Studies on 2 parathyreoprivic patients and one "normal" subject. Am. J. M. Sc., 1941, 202, 691.
70. Aub, J. C., Tibbetts, D. M., and McLean, R., The influence of parathyroid hormone, urea, sodium chloride, fat and of intestinal activity upon calcium balance. J. Nutrition, 1937, 13, 635.
71. Mills, R., Breiter, H., Kempster, E., McKey, B., Pickens, M., and Outhouse, J., The influence of lactose on calcium retention in children. J. Nutrition, 1940, 20, 467.
72. Tibbetts, D. M., and Aub, J. C., Magnesium metabolism in health and disease. I. The magnesium and calcium excretion of normal individuals, also the effects of magnesium, chloride, and phosphate ions. J. Clin. Invest., 1937, 16, 491.
73. a. von Wendt, G., Untersuchungen über den Eiweiss- und Salz-Stoffwechsel beim Menschen. Skandinav. Arch. f. Physiol., 1905, 17, 211.
b. Wiley, F. H., Wiley, L. L., and Waller, D. S., The effect of the ingestion of sodium, potassium, and ammonium chlorides and sodium bicarbonate on the metabolism of inorganic salts and water. J. Biol. Chem., 1933, 101, 73.
74. Watson, E. K., McGuire, E. W., Meyer, F. L., and Hathaway, M. L., Calcium metabolism of pre-school children. J. Nutrition, 1945, 30, 259.
75. Snedecor, G. W., Statistical Methods. Collegiate Press, Ames, Iowa, 1937.

DIFFERENTIAL SPINAL BLOCK. II. THE REACTION OF SUDOMOTOR AND VASOMOTOR FIBERS [1]

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In a previous communication (1), a technic was described for differentially blocking axones as they traverse the subarachnoid space. This was based upon the assumption that the smaller, unmyelinated fibers would be more susceptible to an anesthetic agent in the spinal fluid than the larger, myelinated fibers. At that time, we tested motor power and the patient's appreciation of pin-prick, touch, deep pressure, position sense, and vibratory sense. Skin temperature measurements were used as the index of vasomotor fiber block. It was found that when a 0.2 per cent solution of procaine hydrochloride was used, vasomotor fibers and pin-prick fibers were blocked while motor power and the modalities of touch, deep pressure, position sense, and vibratory sense remained unaffected.

Emmett (2) presented a series of cases in which it was rarely possible to produce a sensory and sympathetic block without a motor block by giving a small volume of procaine solution in the usual high concentrations. Unfortunately, however, the various sensory modalities were not individually examined and the observations on the results of the block were confined to the feet.

Gasser and Erlanger (3), working with cocaine, and Heinbecker, Bishop, and O'Leary (4), working with procaine, have demonstrated that the class C fibers, the least myelinated of a mixed nerve, are the first to be blocked when subjected to local infiltration with the anesthetic agent. It does not necessarily follow that these fibers (in which class autonomic fibers generally fall) are blocked by a lower concentration, but it does suggest that this is so and that, therefore, these fibers could be preferentially blocked.

In this laboratory, preliminary experiments performed on the cat indicated that this was true. A powerful sympathogenic reflex, the carotid sinus reflex, could be abolished in the vagotomized cat by a 0.15 per cent procaine perfusion of the spinal canal [after the method of CoTui (5)], at a time when the animal maintained spontaneous respiratory activity and gave a contralateral muscular response to the electrical excitation of one femoral nerve.

The experiments of Judovich and Bates (6) are interesting in this connection. They demonstrated that varying the concentrations of ammonium chloride surrounding the saphenous nerve of the cat exerted a partially selective effect in obliterating the action potentials of the A and C type fibers. They did not clearly demonstrate that this was not a time gradient as in the work mentioned above. The selective effect of the pitcher plant distillate and ammonium salts, peripherally and intraspinally, in relieving neuralgic pain while leaving the usually tested sensations unimpaired is most stimulating; but the mechanism and fiber types involved are not clear.

This report is concerned mainly with the selective effect, on the basis of a graded concentration, on sudomotor, vasomotor and pin-prick fibers in the subarachnoid space.

MATERIAL AND METHOD

Nine sets of observations were made on seven patients of the Massachusetts General Hospital. Two of these patients were suffering from amputation stump pain, two patients were in the hospital for repair of an inguinal hernia, two for the treatment of herpes zoster, and one for diagnostic procedures relating to sciatic nerve pain. All were otherwise in a good state of health.

Six of the nine sets of observations were concerned with the reactions of the individual to a differential spinal block. The technique used was essentially that as previously described (1). It was somewhat modified in that a larger initial dose (15 ml. rather than 10 ml. of a 0.2 per cent solution) and a higher rate of subsequently administered procaine were used. This was done because it was found that by so doing it was possible to hasten the onset of the differential block without affecting the nature of the response.

[1] Presented at the Ether Day Centenary at the Massachusetts General Hospital on October 15, 1946.

Three sets of observations on the reaction of the patient to the usual or full spinal block were obtained. The manner in which these blocks were performed is specified in the individual protocol. These observations were used as control studies. In two instances both the differential and undifferentiated block were performed in the same patient (R. B. and D. M.).

Iron-constantan thermocouples were placed on the plantar surface of the distal phalanx of the great toe (labelled A in the figures), on the medial aspect of the dorsum of the foot (B), and four inches above the internal malleolus (C). This was done on both lower extremities. A seventh thermocouple (RF) was placed on the palmar surface of the distal phalanx of the right third digit. The eighth thermocouple was placed just under the operating table so that it would register room temperature. The eight thermocouples were connected to a temperature recording device which printed in rotation every 30 seconds. By this means the temperature at each of the designated points was recorded every 4 minutes.[2]

The degree of skin resistance was used to record the order of magnitude of sweating and the changes that occurred. The instrument used was that advocated by Richter (7). The sensitivity of the indicating microammeter, or dermometer, was set at an appropriate level for each individual. This made it impossible to compare the skin resistance and the degree of sweating of one patient with that of another. However the sensitivity setting remained the same for the entire duration of the experiment in any given patient, and, therefore, qualitative changes were accurately reflected. In Figures 2 to 8, the dermometer readings are inversely proportional to the skin resistance.

The patient was brought into a constant temperature room in which the temperature was between 17° and 25° C. In any given experiment the room temperature did not vary more than 1.6° C. The patient's trunk was covered with a cotton sheet; the remainder of the body was bare. The patient was placed supine on the operating table which was maintained in the horizontal position at all times. Blood pressure determinations were done with the mercury manometer, adhering to the criteria of Ragan and Bordley (8). A needle was placed in the subarachnoid space in the third lumbar interspace and connected with tubing which allowed the administration of the 0.2 per cent solution of procaine. This tubing was connected to a leveling bottle which contained the anesthetic solution. A spinal fluid manometer was interpolated by means of a three-way stopcock and held by a clamp so that the zero point was on a level with the skin of the back (Figure 1). The neurologic examination used was that previously described (1). Thermal appreciation was not accurately tested and therefore the results stated do not include this modality. At the suggestion of Dr. J. C. White, tickle was tested in those two patients who were ticklish (E. H. and D. M.), by scratching the soles of their feet.

[2] Brown Instrument Co., Boston, Mass.

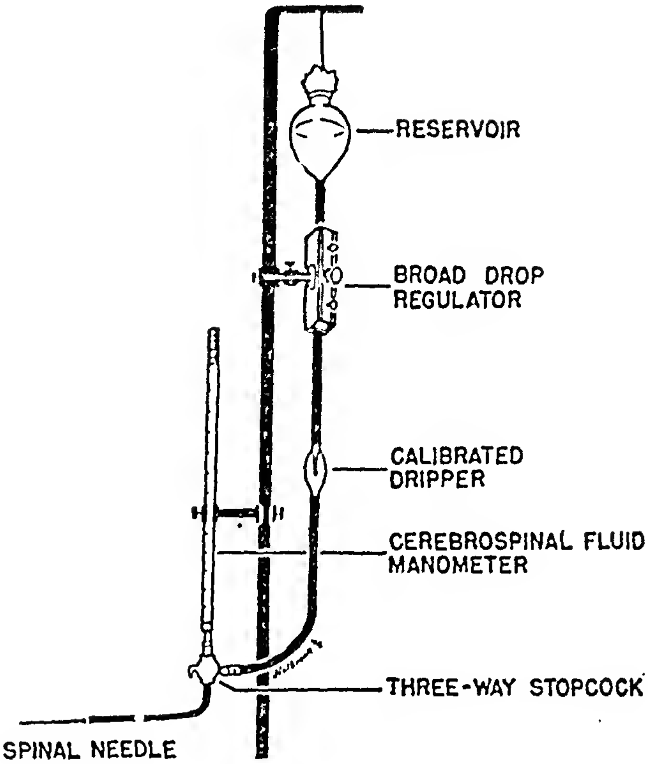

FIG. 1

The results indicate that there is a paralysis of the preganglionic sudomotor fibers which sets in at the same time as the block of the vasomotor fibers. This was true of the differential spinal block as well as of the full spinal block. The difference between the two types of block was mainly in the promptness with which the vasomotor-sudomotor block occurred. It was to be expected in addition that the full spinal block caused the loss of motor power and the other sensory modalities.

Case No. 1 (R. B.): Age 55, male, right inguinal hernia.

(Figure 2A) Satisfactory control skin resistance and skin temperature levels having been obtained, 15 ml. of a 0.2 per cent solution of procaine were administered from 11:37 to 11:42 A.M. 0.6 ml., or 1.2 mgm., per minute were then run in until 12:26 P.M. At 12:00 noon there was evidence of a beginning rise of skin temperature and skin resistance in both lower extremities. The change in the resistance and temperature of the right foot preceded that in the left foot by a few minutes, indicating the similar susceptibility of the two sets of fibers. The skin temperature of both lower extremities soon reached levels indicative of maximal vasodilatation. At 11:54 A.M., there was a spotty loss of the appreciation of pin-prick on the right. This eventually rose to the fifth thoracic segment bilaterally at 12:50 P.M. The remainder of the neurologic examination was negative. The blood pressure

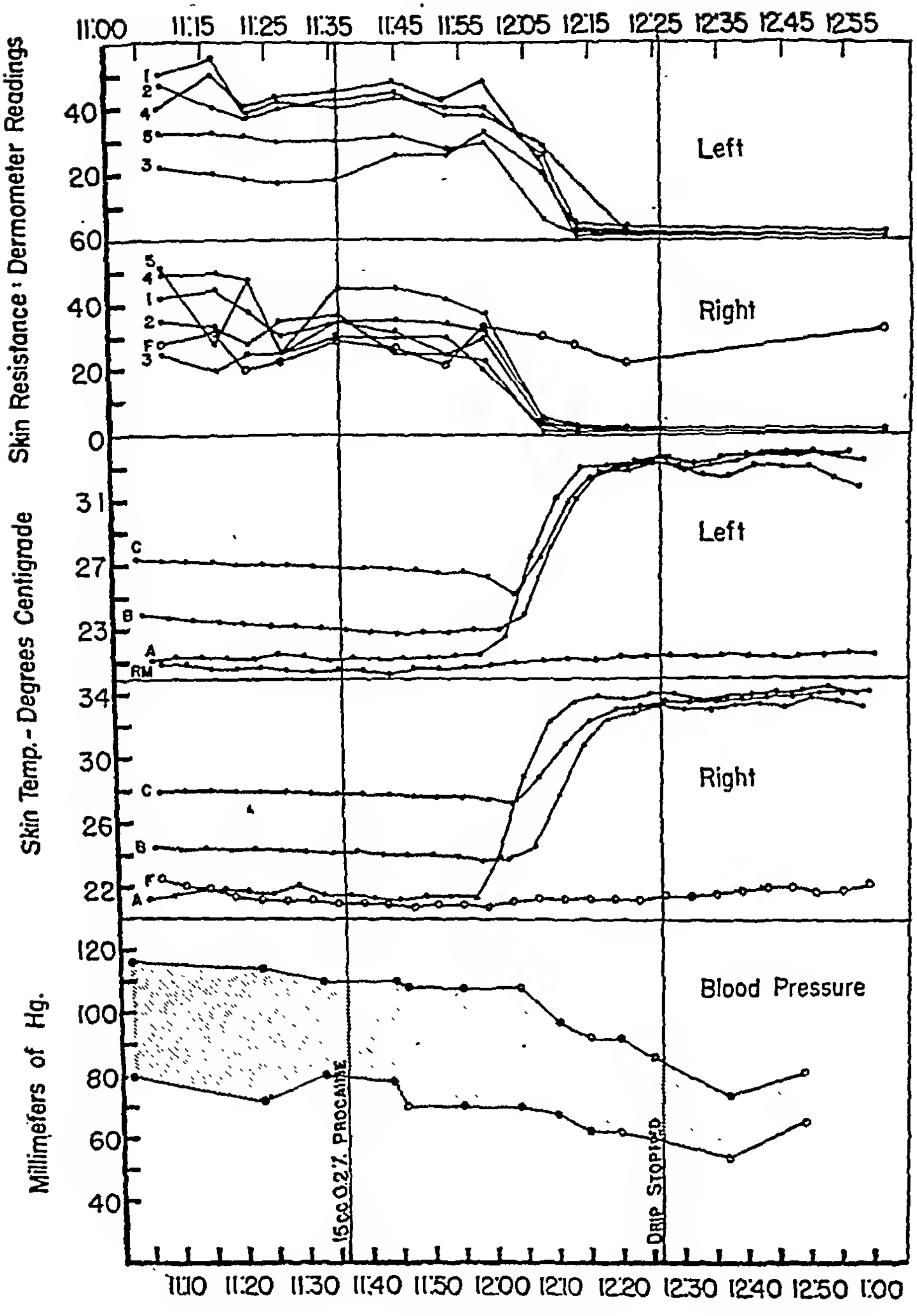

FIG. 2A

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

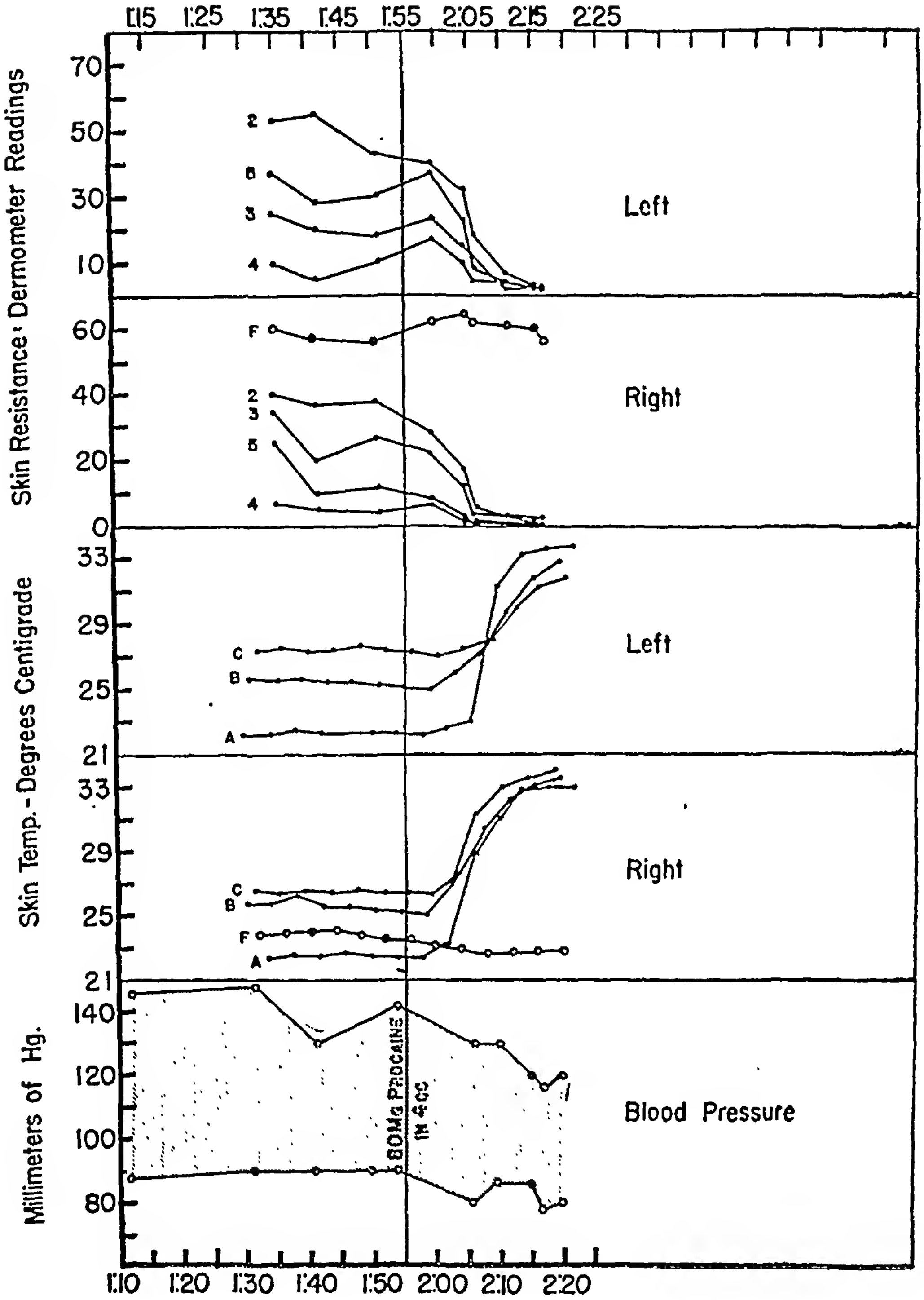

Fig. 2B

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

fell from the control level of 110/80 to a low of 74/54 at 12:37 P.M. A total of 90 mgm. of procaine was administered over a period of 50 minutes. At 12:58, 32 minutes after stopping the drip, a full sudomotor and vasomotor block was still present.

Ten days later, a full spinal block was administered to the same patient (Figure 2B). After control levels were established, 80 mgm. of procaine hydrochloride in 4 ml. of cerebrospinal fluid were injected into the subarachnoid space in the third lumbar interspace. This was followed by the administration of 0.3 ml. per minute of a 0.5 per cent solution of the same agent. A rise in skin temperature and skin resistance in the feet was in evidence within 5 minutes of the injection. The slope of the curve was slightly steeper than when the differential block was administered, but in other respects they were similar. The loss of motor power and sensation was that usually seen with full spinal blocks. The fall in systolic arterial pressure was not so great after the full spinal block as after the differential block (in terms either of absolute or percentile change) despite the fact that muscular paralysis accompanied the former and not the latter.

Case No. 2 (D. M.): Age 38, male. Left mid-thigh amputation stump pain.

(Figure 3A) Control levels having been obtained, 15 ml. of a 0.2 per cent solution of procaine hydrochloride were run in between 3:52 and 3:55 P.M. From then until 4:50 P.M., 1.6 mgm. per minute of the same solution were administered. The skin temperature and skin resistance of the right foot began to rise simultaneously between 4:10 and 4:15 P.M. The skin temperature reached levels indicative of maximal vasodilatation. At 4:10 P.M., there was a just discernible, spotty loss of appreciation of pin-prick on the right anterior thigh. Complete loss of pin-prick sensation eventually rose to the first thoracic segment at 4:51 P.M. At 4:43 P.M., the appreciation of tickle was unchanged. The remainder of the neurologic examination was negative. The blood pressure remained

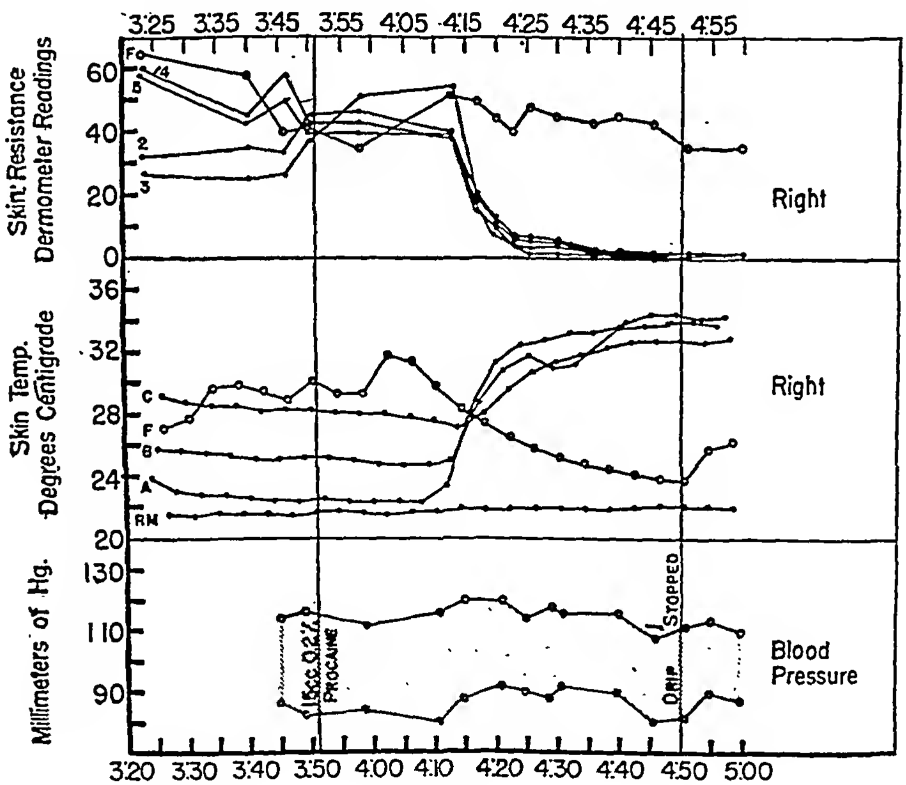

FIG. 3A

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

essentially unchanged. A total of 116 mgm. of procaine was administered over a period of 60 minutes.

Two days later, a full spinal block was administered to the same patient (Figure 3B). After obtaining control levels, 150 mgm. of procaine in 5 ml. of normal saline were injected into the subarachnoid space at the third lumbar interspace. No additional injections were made. This was followed almost immediately by a rise in skin temperature and skin resistance. The curves obtained closely resembled the curves obtained with the differential spinal block in the same patient, with the exception that, with the full block, the skin temperatures did not rise so high during the period of observation. The blood pressure did not change appreciably; but, if anything, the average post-injection level was slightly higher than the average control level.

Case No. 3 (A. D.): Age 68, female. Post-herpetic neuralgia.

(Figure 4) Satisfactory control levels of skin temperature and skin resistance having been obtained, 15 ml. of a 0.2 per cent solution of procaine hydrochloride were run in between 4:35 and 4:39 P.M. From then until 4:58, 1.2 mgm. per minute were administered and from 4:58 to 5:22 P.M., 2.0 mgm. per minute of the same solution were given. The changes in skin temperature and skin resistance in the feet were manifest in the right foot slightly before the left, indicating, as in Case R. B., the similar susceptibility of the two types of fibers to the given concentration of procaine. It is likely that the presence of hypertensive and arteriosclerotic disease limited the height to which the skin temperature rose in this individual. The contour of the curve, however, indicates that the

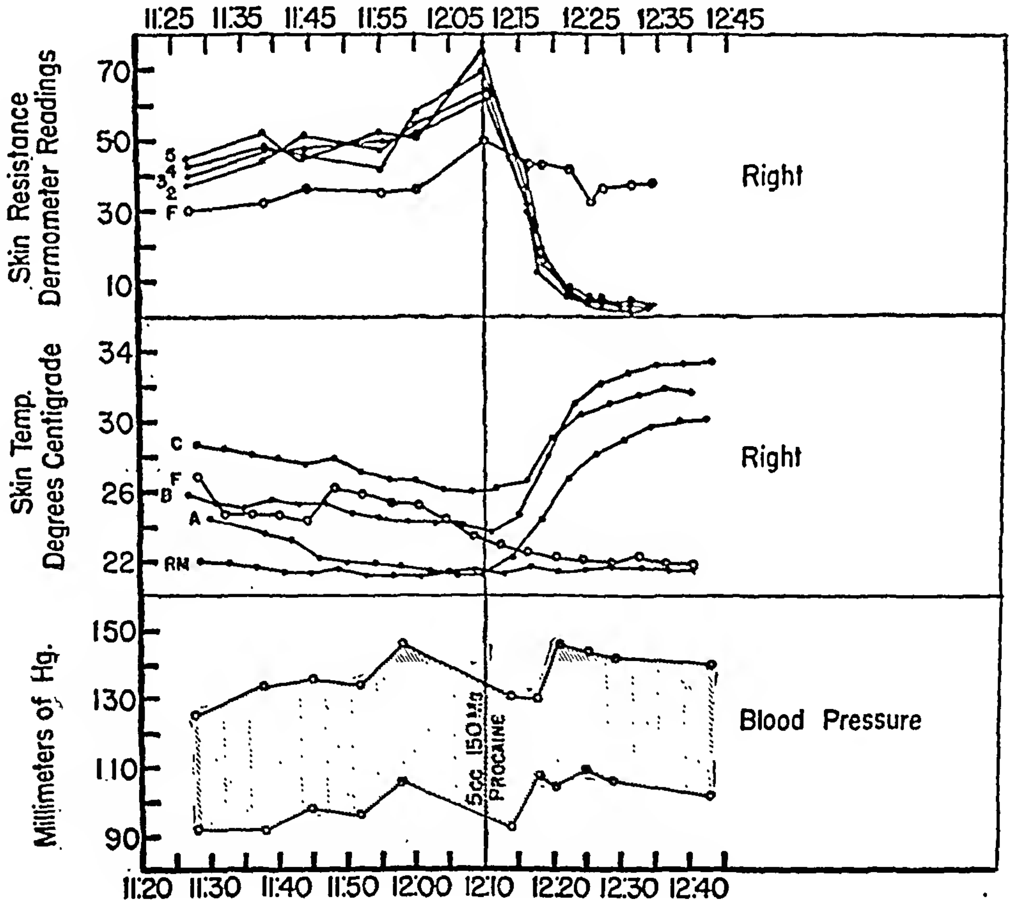

FIG. 3B

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

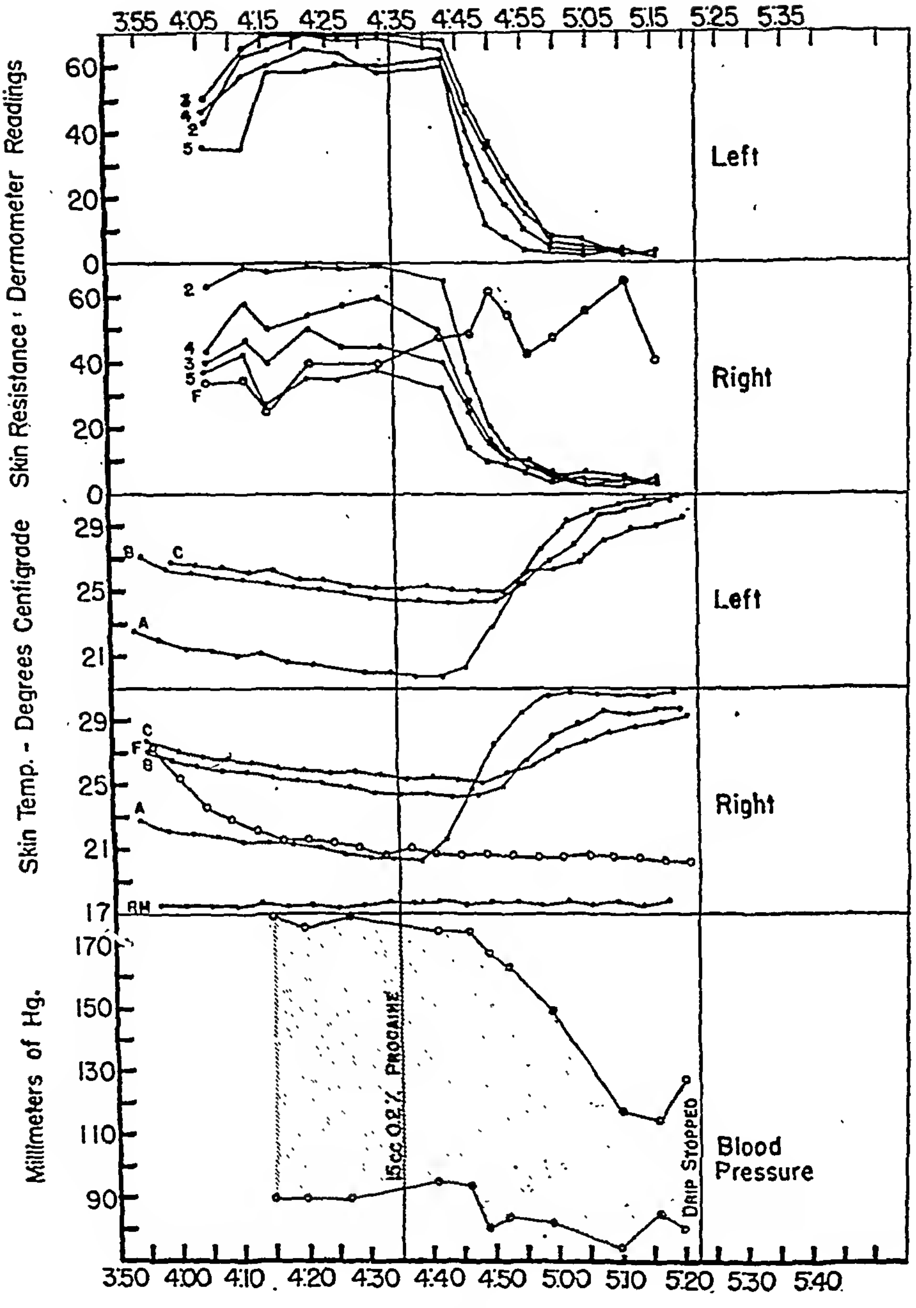

Fig. 4

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

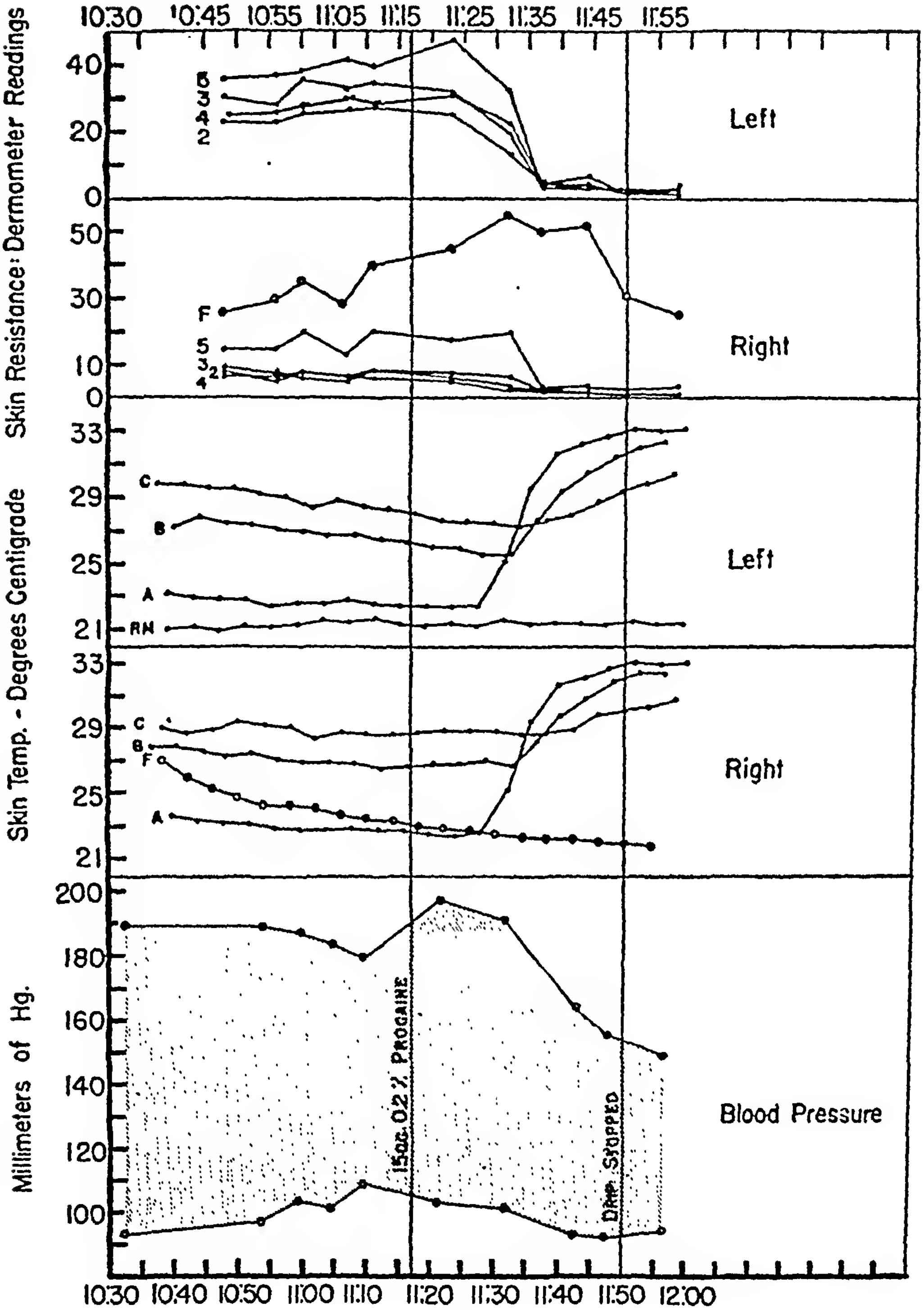

Fig. 5

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

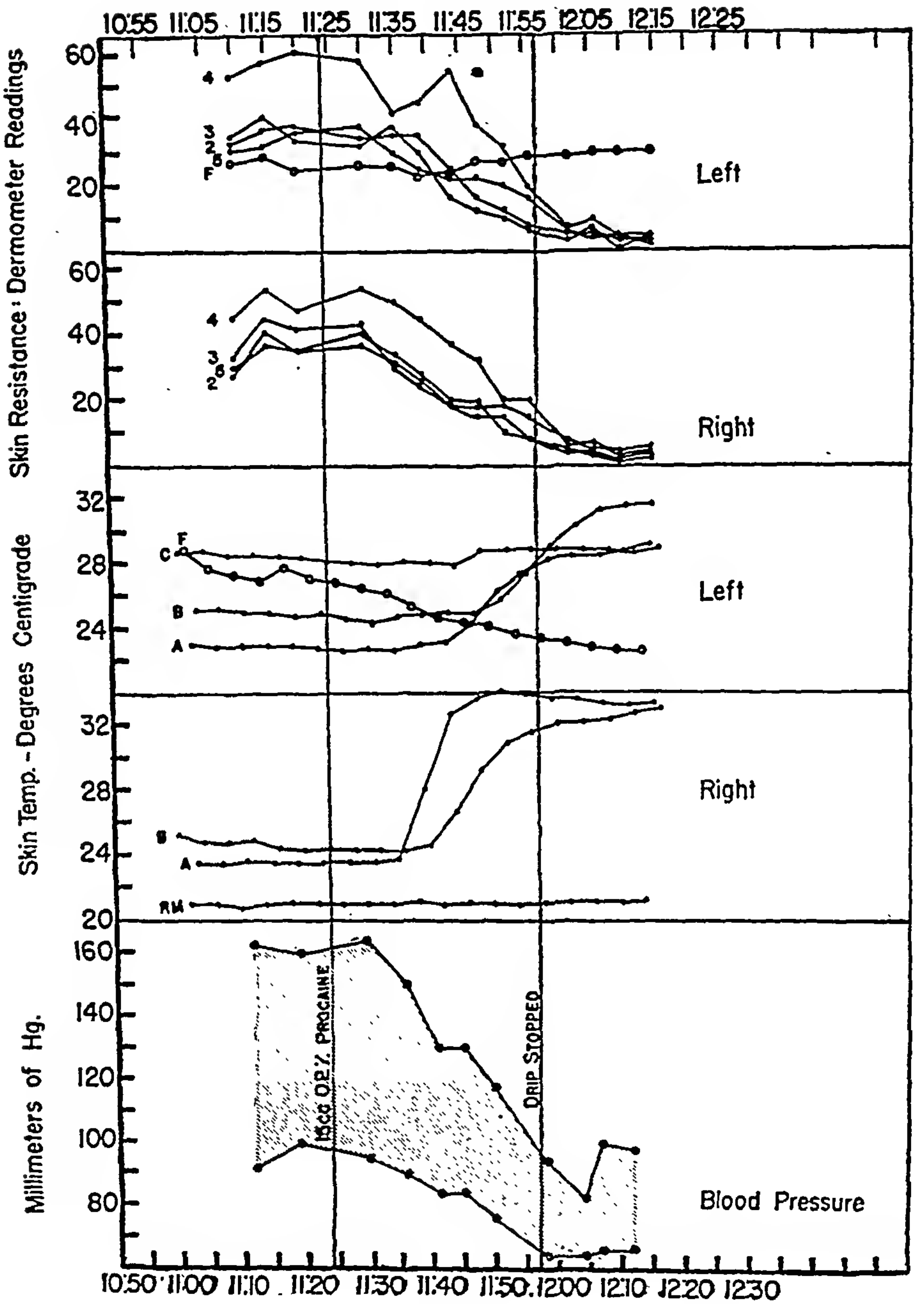

FIG. 6

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

greatest vasodilatation possible in this individual with the use of a chemical block was achieved. At 4:40, there was a patchy loss of the appreciation of pin-prick on the dorsum of both feet. Complete loss of pin-prick sensation eventually rose to the fourth thoracic segment at 5:01 P.M. The remainder of the neurologic examination was negative. The systolic arterial pressure fell from the control level of 178 to 115 mm. Hg. A total of 101 mgm. of procaine was administered over a period of 47 minutes.

Case No. 4 (E. H.): Age 72, female. Amputation stump and phantom pain.

(Figure 5) Previous local nerve section in an attempt to ameliorate the pain in the absent right fifth toe, resulted in high levels of skin resistance in that foot in the control period. The vibratory sense was severely impaired during the control period. After obtaining satisfactory control levels, 15 ml. of a 0.2 per cent solution of procaine were administered between 11:17 and 11:20 A.M. From then until 11:50 A.M., 1.2 mgm. per minute of the 0.2 per cent solution was administered. Between 11:25 and 11:30 A.M. there was evidence of an increase in the skin temperature and skin resistance of the feet. The skin temperature rose to levels indicative of maximal vasodilatation. At 11:27 A.M. there was a beginning loss of appreciation of pin-prick. This eventually rose to the eighth thoracic segment. The remainder of the neurologic examination, including tickle, remained unchanged. The systolic arterial pressure fell from a control level of 185 mm. to 150 mm. Hg. A total of 66 mgm. of procaine was administered over a period of 33 minutes.

Case No. 5 (C. P.): Age 42, male. Differential diagnosis between right sciatic nerve pain and psychoneurosis.

(Figure 6) Satisfactory control levels having been obtained, 15 ml. of a 0.2 per cent solution of procaine were administered from 11:24 to 11:27 A.M. From then until 11:57 A.M., 1.6 mgm. per minute of the same solution were given. At 11:35 A.M. there was evidence of vasomotor and sudomotor block in both lower extremities. The rise in skin temperature of the right foot indicated maximal vasodilatation while that on the left resembled the curve seen with moderate peripheral vascular disease of the occlusive type. There was no dorsalis pedis pulsation to be felt on that side. The slope of the increase in skin resistance was gentler than that seen in the preceding patients. At 11:43 A.M., there was a loss of appreciation to pin-prick at the level of the third lumbar segment and this rose to the second lumbar segment at 11:46. The systolic arterial pressure fell from the control level of 161 to 82 mm. Hg. The remainder of the neurological examination remained unchanged. A total of 75 mgm. of procaine was administered over a period of 33 minutes.

Case No. 6 (J. B.): Age 42, male. Question of post-herpetic neuralgia.

(Figure 7) At the onset of this period of observation, the patient's lower extremities were quite dry and the skin resistance correspondingly high, despite the slightly higher room temperature than that generally used. A higher room temperature could not be used because the skin temperature was already at a level where further elevation would compromise the end-point for vasomotor block. Thus the sensitivity setting of the dermometer had to be set quite high. After obtaining control levels, 15 ml. of a 0.2 per cent solution of procaine were administered between 2:56 and 3:01 P.M. From then until 3:48, 1.6 mgm. per minute of the same solution were given. At 3:04 P.M., there was evidence of a rise in skin temperature and skin resistance in both lower extremities. The skin temperature rose to levels indicative of maximal vasodilatation. The rise in skin resistance was less striking and less regular than in any of the previous patients. It was thought that further drying off of a foot that is already quite dry might yield such a curve. In any case the evidence for a sudomotor block is not so clear as in the other cases observed. At 3:13 P.M., there was a loss of the appreciation of pin-prick to the level of the tenth thoracic segment. This eventually rose to the second thoracic segment at 3:38 P.M. The rest of the neurological examination remained unchanged. A total of 105 mgm. of procaine was administered over a period of 52 minutes.

Case No. 7 (J. M.): Age 21, male. Right inguinal hernia.

(Figure 8) A full spinal block was performed in this patient. He was quite apprehensive, since this procedure was performed immediately prior to operation. All premedication had been omitted for the purposes of this experiment. The room temperature was the highest used for any experiment in this series, and the patient's extremities were all obviously moist. The sensitivity setting of the dermometer thus had to be made quite low in order to bring the needle deflections within the readable scale. At 11:11 A.M., during the control period, the particular surgeon whom the patient knew was to perform the operation, came into the room and announced that they were ready to start. At 11:13 there was a sharp fall in the skin resistance. At 11:17, 100 mgm. of procaine in 4 ml. of cerebrospinal fluid were injected into the subarachnoid space at the third lumbar interspace. This was followed by the administration of 0.4 ml. per minute of a 0.5 per cent solution of the same agent. At 11:21 there was evidence of vasomotor and sudomotor block. The skin temperature of the feet rose to levels indicative of maximal vasodilatation, and the skin resistance rose sharply. The patient was not followed closely thereafter, but inasmuch as the operative procedure was completed without discomfort to the patient and with adequate muscular relaxation, it is likely that the full block was that as usually seen. At the end of the period of observation (11:32) the patient's blood pressure had risen about 5 mm. Hg.

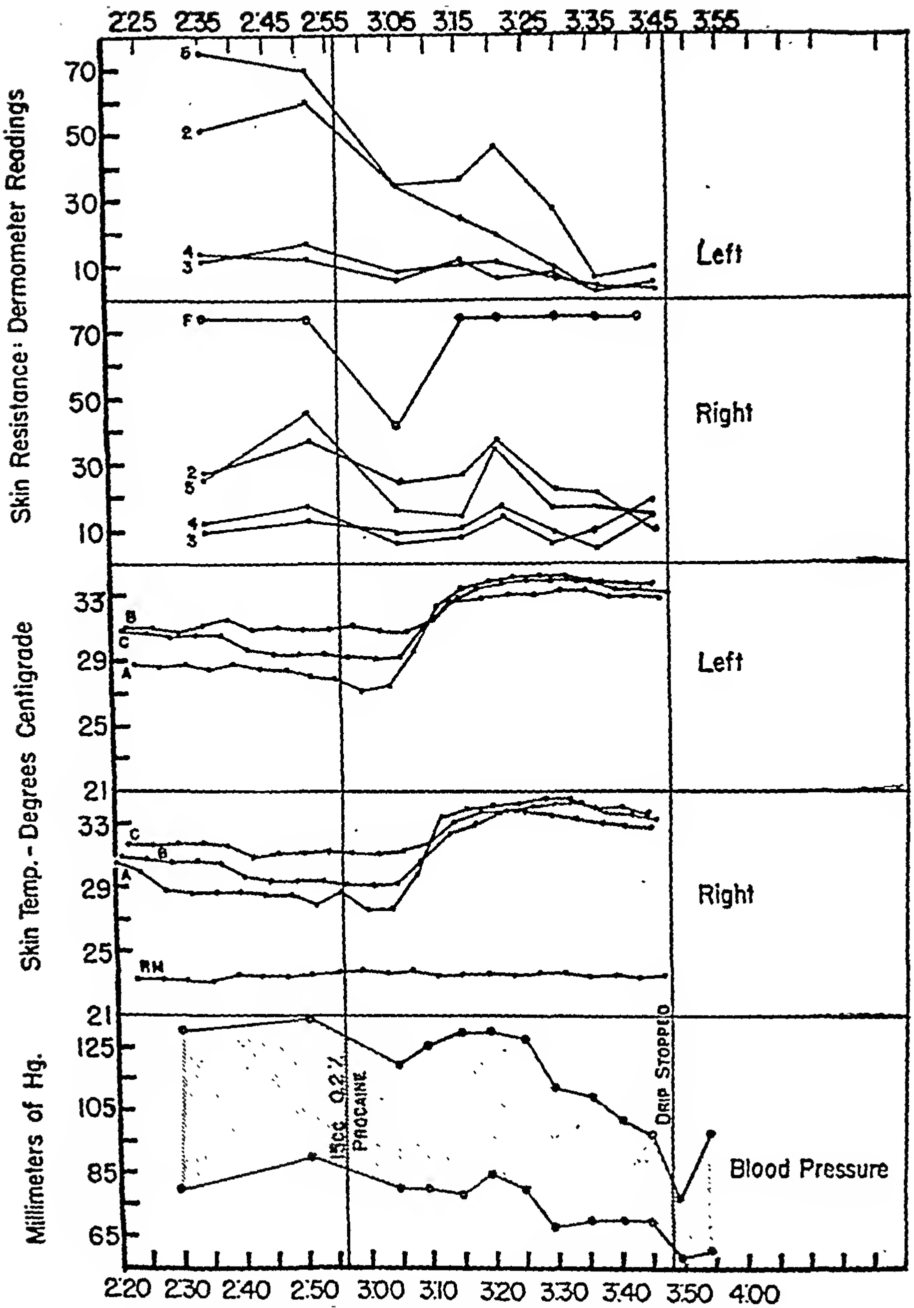

FIG. 7

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

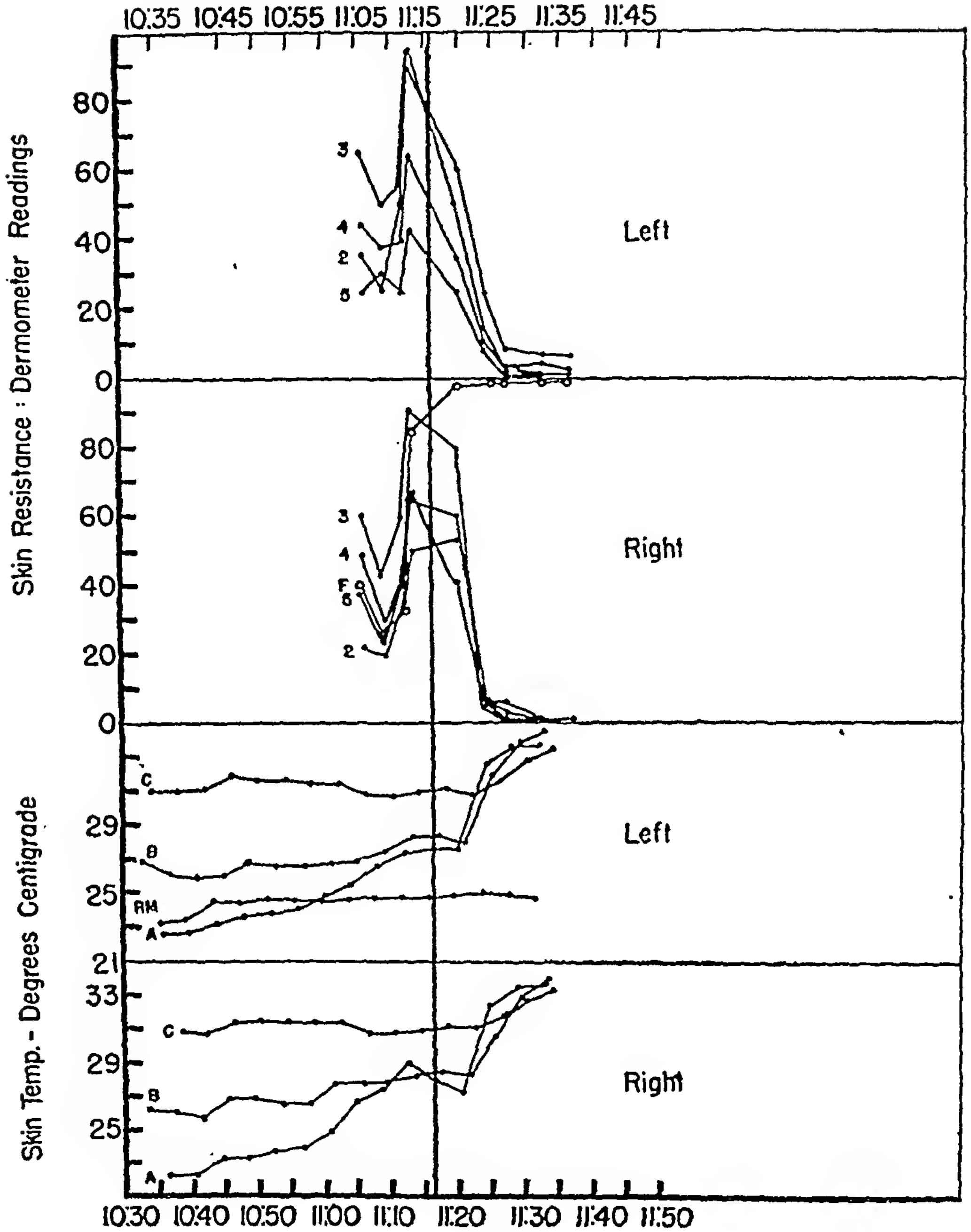

FIG. 8

Under skin resistance, the number indicates the toe examined. The plantar surface of the distal phalanx was used in each case. F indicates the readings obtained from the palmar surface of the distal phalanx of the fourth right finger. The dermometer readings are inversely proportional to the skin resistance. Under skin temperature, A represents the readings obtained from the plantar surface of the distal phalanx of the great toe, B from the medial aspect of the dorsum of the foot, and C from the inner aspect of the leg four inches above the internal malleolus. F represents readings from the palmar surface of the distal phalanx of the third right finger.

DISCUSSION

The data just presented indicate that preganglionic sudomotor fibers are blocked by the concentration of procaine which results from the dilution of the 0.2 per cent solution introduced in the amounts stated above. They are apparently blocked simultaneously with the preganglionic vasomotor fibers and either preceded or followed by a block of fibers concerned with the appreciation of pin-prick. This concentration of the

agent leaves unaffected the fibers, mediating position sense, vibratory sense, touch, deep pressure, and motor power, insofar as this is reflected by the neurological examination used. The data presented here may be considered confirmatory of the preliminary report recently published (1).

In the two patients of this series in whom tickle was tested, the sensation was unimpaired at a time when there was a complete loss of the appreciation of pin-prick. Three subsequent patients have been studied in regard to this modality. In one of these there was no impairment, in one there was partial impairment, and in one there was complete loss. It would seem therefore, that although this modality is distinct from that of appreciation of pin-prick, the fiber that subserves it is more nearly like that of the pain fiber in size and myelination than are the fibers subserving motor power, position sense, touch, and vibratory sense.

The degree of myelination and fiber size of the various components of spinal nerves have been reviewed by Ransom (13). He states that efferents to skeletal muscles are myelinated fibers, which are, for the most part, of large caliber. Touch is mediated by larger myelinated fibers, while pain is mediated by the fine myelinated or unmyelinated fibers. It is also stated that proprioceptive fibers are myelinated. Sympathetic fibers (efferent) are all finely myelinated (14).

If the assumption is made that the centrally directed axones from the spinal ganglia are similar to those of the peripheral axones, the data herewith presented would seem to be in accord with accepted morphologic data. One important exception has been noted (11) in that it has been found that the knee kicks and ankle jerks disappear at a time when position sense is unimpaired. This would make it seem that the stretch afferents differ from other proprioceptive fibers in that they are relatively small unmyelinated fibers. It should be emphasized that these data are significant only for the subarachnoid portion of the spinal nerves.

It was previously stated that a differential block, without motor loss, induced a degree of hypotension similar to that seen in full or undifferentiated spinal block (1). This was interpreted as meaning that muscular paralysis and the venular stasis that supposedly follows it contribute little to the hypotension of spinal anesthesia in the horizontal position. With one exception, the falls in arterial pressure seen in the patients of this series subjected to a differential block were of the same order of magnitude as those seen after full spinal block. This one exception (Case D. M.) failed to have a fall with a full spinal block as well, and indeed, had a slight rise. The nature of this patient's response is not clearly understood.

The technic of differential spinal block is not to be construed as an anesthetic procedure. Rather, it has proved useful in the investigation of problems concerned with the peripheral nervous system such as amputation stump and phantom limb pain (9). The technic has yielded interesting information in patients with intestinal dyskinesia and colonic atony. Since somatic motor paralysis is not present, one may introduce amounts of procaine solution high enough in the spinal canal to be sure of blocking all visceral efferents to the intestinal tract (10).

In a recently concluded study, the knee kick, ankle jerk and abdominal reflexes were examined in twelve patients under the influence of a differential spinal block as described above. It had previously been thought that position sense and the stretch afferents were, by and large, subserved by the same group of proprioceptive afferent fibers. That this is not the case is indicated by the fact that these reflexes were abolished in every case while position sense remained unchanged (11).

It had previously been demonstrated that the undifferentiated type of spinal block confers benefit upon the patient suffering from pulmonary edema due to cardiac decompensation (12). It was thought that the arteriolar dilatation would diminish the resistance against which the left ventricle would have to work, and also that the pooling of blood in the periphery would significantly diminish the return flow to the right heart. The drawback to the procedure as it was practised at that time is that a partial intercostal paralysis accompanied the peripheral pooling effect. This was felt to be hazardous in the dyspneic patient in need of full respiratory excursions. The technic of differential spinal block, however, allows one to induce the peripheral vasodilatation without the intercostal paralysis. Two patients have been so

treated and responded with a convincing clinical improvement. This was temporary in one. In one of these patients arterial and venous oxygen determinations were performed and clearly confirmed the clinical impression.

Previous data (1) demonstrated that it was possible to produce a chemical sympathectomy of the upper as well as the lower extremity by means of a differential spinal block. From the above data it can be seen that the degree of vasodilatation induced with the differential spinal block is equivalent to that induced with a full spinal block. It should therefore be useful as a prognostic technic in patients with peripheral vascular disease.

At the present time a differential spinal block is performed almost routinely preoperatively in hypertensive patients. Since the technic is essentially a reversible sympathectomy, an attempt will be made to ascertain whether or not the fall in blood pressure induced by the block will correspond to that accomplished by sympathectomy.

An interesting but as yet unexplained side effect noticed in about one-third of patients is the observation that they become distinctly somnolent at a time when procaine is high in the spinal canal.

If the basic assumption is correct, and we believe it to be so, that relative susceptibility to graded concentrations of the anesthetic agent depends upon fiber size and degree of myelination, then the above data are indicative of the nature of the preganglionic sudomotor fibers. Such a conclusion, based upon this type of clinical evidence, is in agreement with the early observations of Gaskell (14) who demonstrated that preganglionic sympathetic efferents were morphologically indistinguishable from one another.

SUMMARY

1. Preganglionic sudomotor fibers in the subarachnoid space are blocked by the introduction of appropriate amounts of 0.2 per cent procaine hydrochloride.

2. The onset of the sudomotor block is simultaneous with the paralysis of the preganglionic peripheral vasoconstrictor fibers. This is either preceded or followed by a block of fibers concerned with the appreciation of pin-prick.

3. The preganglionic sudomotor fibers are susceptible to a concentration of anesthetic agent which leaves motor, touch, deep pressure, vibratory sense, and position sense fibers intact.

4. Fibers subserving the tickle sensation are generally unaffected and are distinct from those mediating pain.

5. The rôle of muscular paralysis in the genesis of the hypotension seen with spinal anesthesia must be very limited.

BIBLIOGRAPHY

1. Sarnoff, S. J., and Arrowood, J. G., Differential spinal block. A preliminary report. Surgery, 1946, 20, 150.
2. Emmett, J. L., Subarachnoid injections of procaine hydrochloride; quantitative effects of clinical doses on sensory, sympathetic and motor nerves. J. A. M. A., 1934, 102, 425.
3. Gasser, H. S., and Erlanger, J., Role of fiber size in establishment of nerve block by pressure or cocaine. Am. J. Physiol., 1929, 88, 581.
4. Heinbecker, P., Bishop, G. H., and O'Leary, J., Analysis of sensation in terms of nerve impulse. Arch. Neurol. and Psychiat., 1934, 31, 34.
5. Co Tui, Burstein, C. L., and Ruggiero, W. F., Total spinal block; a preliminary report. Anesthesiology, 1940, 1, 280.
6. Judovich, B., and Bates, W., Segmental Neuralgia in Painful Syndromes. F. A. Davis Company, Phila., 1944.
7. Richter, C. P., and Otenasek, F. J., Thoracolumbar sympathectomies examined with the skin resistance method. J. Neurosurg., 1946, 3, 120.
8. Ragan, C., and Bordley, J., III, Accuracy of clinical measurements of arterial blood pressure, with note on auscultatory gap. Bull. Johns Hopkins Hosp., 1941, 69, 504.
9. Arrowood, J. G., and Sarnoff, S. J., Differential spinal block. V. Investigation of the peripheral nerve fibers subserving amputation stump and phantom limb pain. (Manuscript in preparation.)
10. Sarnoff, S. J., Arrowood, J. G., and Chapman, W. G., Differential spinal block. IV. Investigation of intestinal dyskinesia, colonic atony and visceral afferents. (Manuscript in preparation.)
11. Sarnoff, S. J., and Arrowood, J. G., Differential spinal block. III. The abolition of cutaneous and stretch reflexes in the presence of unimpaired position sense. J. Neurophysiol. (In press.)
12. Sarnoff, S. J., and Farr, H. W., Spinal anesthesia in the therapy of pulmonary edema. A preliminary report. Anesthesiology, 1944, 5, 69.
13. Ranson, S. W., The Anatomy of the Nervous System. W. B. Saunders & Co., Phila. 7th edition, 1943. Chapter 5.
14. Gaskell, W. H., On the structure, distribution, and function of the nerves which innervate the visceral and vascular systems. J. Physiol., 1886, 7, 1.

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would throw light on some of the ... symptoms and signs which occur during and following therapy for diabetic acidosis.

MATERIAL

Fourteen patients, ranging from ages 12–69, who entered the hospital in severe diabetic acidosis (carbon-dioxide combining power expressed as milliequivalents of bicarbonate, under 9 milliequivalents) were studied. All of these patients survived with the exception of a 56-year-old woman who died within 30 hours, with unexplained hyperpyrexia and morbilliform rash. Post-mortem examination did not contribute to the solution of the problem, although virus studies are not yet completed. Serial determinations of serum potassium, sodium, calcium, magnesium, and total protein were made on all patients, in addition to the usual determinations of blood sugar, carbon-dioxide combining power, and non-protein nitrogen. In seven of the patients pH, and albumin and globulin fractions were also determined. Phosphorus levels determined as inorganic phosphate were run on two patients.

METHODS

The pH was determined on venous blood by the use of the Beckman pH meter, serum sodium by a modification of the zinc uranyl acetate method of Barber and Kolthoff

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...the method of Benedict (14).

...calculated from the mass action

$$\frac{(\ldots\text{ate}^{=})}{(\text{Calcium Proteinate})} = 10^{-2.22}$$

(pK Calcium Proteinate = 2.2) (15).

Total protein was converted to grams per 1000 grams H_2O by the formula:

grams water per 100 ml. = 99–0.75 × protein (grams per 100 ml.) (16)

and albumin and globulin fractions corrected accordingly.

The base combining capacity of the albumin and globulin were calculated at each pH, using the values of Van Slyke, *et al.* (17).

M.eq. Base per grams albumin = 0.125 (pH − 5.16).

M.eq. Base per grams globulin = 0.077 (pH − 4.89).

RESULTS

Table I shows the changes in electrolytes, blood sugar, carbon-dioxide combining power, and non-protein nitrogen, correlated with treatment (amount of insulin, fluids, sodium, and glucose), and with the clinical state.

Certain general features appear from inspection of this table:

1. The marked acidosis, and hemoconcentration on entry. The total protein concentration was used as a gauge of hemoconcentration.
2. The fall in serum potassium concentration during therapy. In some patients the values reached critically low levels—1.9 and 2.18 M.eq.

[1] Since the preparation of this manuscript a report of 1 patient in diabetic acidosis who developed low potassium levels (2.5 milliequivalents) during therapy, which were associated with respiratory paralysis, and which responded to potassium intravenously, has appeared. Holler, Jacob W., Potassium deficiency occurring during the treatment of diabetic acidosis. J. A. M. A., 1946, 13, 1186.

TABLE I

Correlation of chemistry, treatment and clinical state

Time: Day	Time: Hr.	Blood chemistry					Serum protein: Alb.	Serum protein: Glob.	Serum protein: Total	Sugar	HCO_3^-	NPN	Treatment: Therapy between hrs. listed, with 24 hr. summary(1): N.S.(2)	M/6 Na lactate	5% glucose in N.S.	5% glucose in H_2O	Plasma	Insulin	Clinical state
		pH	K *meq.*	Na *meq.*	Ca *meq.*	Mg *meq.*	*grams per cent*	*grams per cent*	*grams per cent*	*mgm. per cent*	*meq.*	*mgm. per cent*	*liters*	*liters*	*liters*	*liters*	*liters*	*units(3)*	
No. 1, Patient T. B., Colored Female, 19, No. 683-113																			
1	0	7.05	6.72	152	5.25	1.98	5.9	3.4	9.7	545	7.5								
	6	7.29	5.42	161	4.65	1.16	4.7	2.6	7.3	230	9.5		2		1			110	Conscious
	21									117	22.0			2		.65 (10%)		90	
	24																	30	
													5,650 ml. fluids; 18.3 grams Na; 115 grams glucose; 230 units insulin						
2		7.55	3.20	158	4.80	0.56	3.9	2.0	5.9									80	Muscle aches
3		7.55	4.56	160	4.70	1.40	3.6	1.9	5.5									60	
4		7.58	4.56	160	5.00	1.48	4.1	2.1	6.2									60	
No. 2, Patient A. B., White Male, 69, No. 723-522																			
1	0									618	8.0	65							
	2	7.28	5.38	138	4.35	1.47	3.5	2.3	5.8	452	10.0	48	1					80	Conscious
	20	7.60	1.90	142	4.30	1.06	3.1	1.8	4.9	97	24.0		1.3 Gl.(4)		1	2		225	
	24																	15	
													5,350 ml. fluids; 11.8 grams Na; 175 grams glucose; 320 units insulin						
2	1	7.65	2.77	142	4.40	1.39	3.0	1.9	4.9									35	Marked weakness
	20	7.63	2.18	152	4.30	1.47	3.1	1.9	5.0										
3		7.59	3.36	153	4.25	1.39	3.0	1.9	4.9									20	
4		7.60	5.13	151	4.45	1.56	3.1	2.1	5.2									30	
5		7.57	6.64	145	4.40	1.23	2.8	1.8	4.6									30	
6		7.52	4.31	156	4.75	1.31	3.5	2.2	5.7									60	
7		7.55	6.13	146	4.50	1.39	3.2	1.9	5.1									60	
No. 3, Patient H. C., White Female, 56, No. 964-879																			
1	2	7.10		147	4.90	2.75	5.1	2.9	8.0	830	<4.5	38							Deeply comatose; shock
	23	7.22	4.32	163	5.30	1.83	4.4	2.4	6.8	204	19.0	45	2	1	3		1	205	
													7,000 ml. fluids; 21.5 grams Na; 150 grams glucose; 205 units insulin						Death

(1) Includes only intravenous fluids given between the hours listed.
(2) Isotonic saline.
(3) Insulin in first 24 hours was crystalline zinc insulin given in divided doses every 1-3 hours. On remaining days total includes crystalline zinc and protamine zinc insulin.
(4) 50 ml. 50 per cent glucose in water.

TABLE I—*Continued*

Time		Blood chemistry											Treatment						Clinical state
							Serum protein						Therapy between hrs. listed, with 24 hr. summary(1)						
Day	Hr.		K	Na	Ca	Mg	Alb.	Glob.	Total	Sugar	HCO_3^-	NPN	N.S.(2)	M/6 Na lactate	5% glucose in N.S.	5% glucose in H_2O	Plasma	Insulin	
		pH	*meq.*	*meq.*	*meq.*	*meq.*	*grams per cent*	*grams per cent*	*grams per cent*	*mgm. per cent*	*meq.*	*mgm. per cent*	*liters*	*liters*	*liters*	*liters*	*liters*	*units(3)*	
No. 4, Patient E. S., White Female, 19, No. 962–393																			
1	2									870	<4.5								Semi-comatose; shock
	10	7.32	4.26	150	5.20	1.56	4.3	1.9	6.2	302	4.9	55	1.4	1	1		0.5	430	
	13	7.49	2.20	150	5.00	1.31	4.3	1.9	6.2	172	16.5		0.35	1 & Gl.(5)		Gl.(4) Gl.(4)	0.5	70	
	16	7.64	2.18	150	5.15	1.39	4.3	1.8	6.1	335	23.0							100	
	24									107	21.5				1			120	
													6,950 ml. fluids; 20.9 grams Na; 200 grams glucose; 720 units insulin						
2		7.51	3.85	164	4.90	1.88	3.9	1.8	5.7	130	17.5		1		1 (10%)	Gl.(4)		160	Muscle stiffness
3										372	17.0	61	1		1			90	Muscle weakness
4		7.32	3.49	155	3.55	1.15	3.5	1.6	5.1									70	Muscle weakness
5			3.67	154	3.95	1.15	3.5	1.8	5.3	400		35						180	
6										83	28.5							?	
8			4.66	154	4.15	0.94	4.0	2.4	6.4									165	Weakness
37		7.33	6.18	155	4.95	1.39	5.2	2.7	7.9									120	
No. 5, Patient B. M., White Male, 19, No. 968–418																			
1	2	7.12	7.28	174	5.70	2.13	6.8	3.0	9.8	575	7.0								Conscious
	5	7.45	5.46	165	5.10	1.72	5.7	2.4	8.1	215	10.0		1	1 1 & Gl.(6)				105	
	9	7.51	5.00	159	4.65	1.56	4.5	1.9	6.4	227	18.5				1			40	
	15			144	4.40	1.80	4.1	1.8	5.9	84	23.5				2			30	
	24																	0	
													6,150 ml. fluids; 21.8 grams Na; 225 grams glucose; 175 units insulin						
2		7.58	5.74	140	4.40	1.23	4.1	1.8	5.9									100	
5		7.51	5.64	142	4.85	1.47	4.4	2.1	6.5	252	26.0							90	
No. 6, Patient N. W., White Female, 44, No. 319–307																			
1	0		8.72	133	4.95	1.88	4.7	3.2	7.9	683	<4.5								Conscious
	20	7.58	3.41	141	3.85	0.82	3.1	2.0	5.1	178	17.0		2(8) 2	1.3	1	1		110	
	24																	0	
													7,300 ml. fluids; 22.7 grams Na; 100 grams glucose; 110 units insulin						
2	1	7.65	7.61	139	4.00	1.23	3.0	1.8	4.8									25	
	23	7.60		135	4.05	1.23	3.1	2.0	5.1										
5		7.56	5.64	153	4.50	1.47	3.9	2.3	6.2									35	
7		7.38	6.14	153	3.95	1.31	3.5	2.2	5.7	370 570 330	7.5 11.5 17.5							95	
8			5.28	153	4.15	1.31	3.4	2.2	5.6									60	
11			4.59	153	4.55	1.56	3.7	2.3	6.0									40	

(5) 100 ml. 50 per cent glucose in water added to the 1/6 M sodium lactate.
(6) 150 ml. 50 per cent glucose in water added to the 1/6 M sodium lactate.

TABLE I—*Continued*

No. 7, Patient T. A., White Male, 17, No. 34-034

Time		Blood chemistry										Treatment: Therapy between hrs. listed, with 24 hr. summary(1)						Clinical state
						Serum protein												
Day	Hr.	K	Na	Ca	Mg	Alb.	Glob.	Total	Sugar	HCO_3^-	NPN	N.S.(2)	M/6 Na lactate	5% glucose in N.S.	5% glucose in H_2O	Plasma	Insulin	
		meq.	meq.	meq.	meq.	grams per cent	grams per cent	grams per cent	mgm. per cent	meq.	mgm. per cent	liters	liters	liters	liters	liters	units(3)	
1	0	6.00	148	4.90	2.33	5.2	3.1	8.3	375	<4.5	37							Semi-comatose
	4	5.57	148	4.15	1.83	4.3	2.4	6.7	235	6.5		2		1			120	
	8								180	21.0								
	16		161	4.25	1.83	3.7	2.2	5.9	119				1	1			90	
	24																15	
												5,000 ml. fluids; 18 grams Na; 100 grams glucose; 225 units insulin						
2		4.36	154	4.00	0.98	3.2	1.6	4.8									60?	
3		5.93	159	4.40	1.23	3.5	2.0	5.5									60?	

No. 8, Patient A. D., White Female, 39, No. 954-618

Day	Hr.	K	Na	Ca	Mg	Alb.	Glob.	Total	Sugar	HCO_3^-	NPN	N.S.(2)	M/6 Na lactate	5% glucose in N.S.	5% glucose in H_2O	Plasma	Insulin	Clinical state
1	0								285	6.5								
	3	4.26	151	4.40	1.11			7.9	352	12.5		1	1 & Gl.(5)				80	Conscious
	15								192	23.5			1 & Gl.(5)	2	3		275	
	24															0.5	40	
												8,700 ml. fluids; 18.3 grams Na; 350 grams glucose; 395 units insulin						
2		7.21	151	4.55	1.31			5.8									30	

No. 9, Patient M. K., White Male, 16, No. 947-988

Day	Hr.	K	Na	Ca	Mg	Alb.	Glob.	Total	Sugar	HCO_3^-	NPN	N.S.(2)	M/6 Na lactate	5% glucose in N.S.	5% glucose in H_2O	Plasma	Insulin	Clinical state
1	0								570		54							Conscious
	2		150	5.25	2.21				400	5.5		1					140	
	21		140	4.95	1.39				270	20.5	37	3.15	1 & Gl.(5)		2 1(7)	0.55	225	
	24																40	
												8,800 ml. fluids; 18.5 grams Na; 200 grams glucose; 405 units insulin						
7		5.84	154	5.20	1.72												85	

No. 10, Patient F. C., White Male, 12, No. 211-641

Day	Hr.	K	Na	Ca	Mg	Alb.	Glob.	Total	Sugar	HCO_3^-	NPN	N.S.(2)	M/6 Na lactate	5% glucose in N.S.	5% glucose in H_2O	Plasma	Insulin	Clinical state
1	0								1,092	<4.5								Deeply comatose; shock
	3		141	5.25	2.46			7.2	810	9	121	0.6	0.15			0.5	200	
	6		141	5.75	2.87			7.2	487	18.5	150		0.5			0.25	90	
	24											1		0.65	0.13		185	
												3,780 ml. fluids; 10.5 grams Na; 39 grams glucose; 475 units insulin						
2									98	16.5		1		0.60	0.67		70	
3									219	18.5		0.3		1	2.80		55	
4		4.87	136	4.45	1.56			5.6	282	8.0	129			1	1		50	
5		8.15	146	4.30	2.04			5.2	476	19.0	99			1	1		120	

(7) $2\frac{1}{2}$ per cent glucose in water given subcutaneously.

TABLE I—*Continued*

No. 10, Patient F. C., White Male, 12, No. 211–641—(*continued*)

Time		Blood chemistry											Treatment						Clinical state
							Serum protein						Therapy between hrs. listed, with 24 hr. summary(1)						
Day	Hr.		K	Na	Ca	Mg	Alb.	Glob.	Total	Sugar	HCO_3^-	NPN	N.S.(2)	M/6 Na lactate	5% glucose in		Plasma	Insulin	
		pH	*meq.*	*meq.*	*meq.*	*meq.*	*grams per cent*	*grams per cent*	*grams per cent*	*mgm. per cent*	*meq.*	*mgm. per cent*	*liters*	*liters*	*N.S. liters*	*H_2O liters*	*liters*	*units(3)*	
7										770	10.5	100			1	1		85	
10			4.26	157	3.95	1.06			4.9			35				1		60	
21			7.2	147	4.80	1.35			6.2									80	
38			5.87	150	5.50	1.39	4.8	2.8	7.6	218								70	
No. 11, Patient J. S., White Female, 18, No. 866–215																			
1	0			149	5.50	2.46				500	5.5	27							Conscious
	20			147	3.85	1.52				48	21.5		2.35	1 & Gl.(5)	2	2		190	
	24																	20	
													7,450 ml. fluids; 19.2 grams Na; 250 grams glucose; 210 units insulin						
5				144	5.65	1.23												80	
No. 12, Patient R. B., White Male, 40, No. 950–958																			
1	0									1,092	5.5	95							Comatose; shock
	2		5.11	150	5.55	2.95			8.6	1,260	<4.5		2				0.25	180	
	24									310	20.5	40	4.7	1 & Gl.(5)		3 1(7)	0.50	820	
													12,550 ml. fluids; 27.5 grams Na; 225 grams glucose; 1,000 units insulin						
6			4.98	149	4.45	1.39			6.7									80	
13			5.54	141	4.65	1.23			5.5									65	
No. 13, Patient J. W., White Male, 13, No. 949–854																			
1	0			139	5.15	1.56			7.2	309	<4.5	44							Conscious
	19		3.52	144	5.45	1.23			6.1	175	14.0		1		4	2		480	
	24																	40	
													7,000 ml. fluids; 17.7 grams Na; 300 grams glucose; 520 units insulin						
6			5.67	142	4.85	1.31			6.1									75	
No. 14, Patient E. R., White Male, 40, No. 949–632																			
1	1		10.34	143	5.05	2.75				950	<4.5	77							Semi-comatose; shock
	20		3.82	157	4.30	0.98			5.4	326	19.5		3.5 1(8)	1	1	1.2	1	480	
	24														1	1		40	
													10,700 ml. fluids; 26.8 grams Na; 210 grams glucose; 520 units insulin						
8			5.08	143	4.40	1.48			5.9									100	

(8) Normal (isotonic) saline given subcutaneously.

3. Slightly low to normal sodium levels on entry, indicating with the hemoconcentration decreased total circulating sodium.

The relative constancy of the sodium concentrations during therapy, despite the use of large amounts of sodium intravenously—10.5–27.5 grams.

4. The magnesium levels on entry were normal or elevated. There was a striking fall of concentration with the therapy of the first 24 hours. Values as low as 0.56–0.82 M.eq. were obtained.

5. The majority of the calcium concentrations fell within the normal range, although four

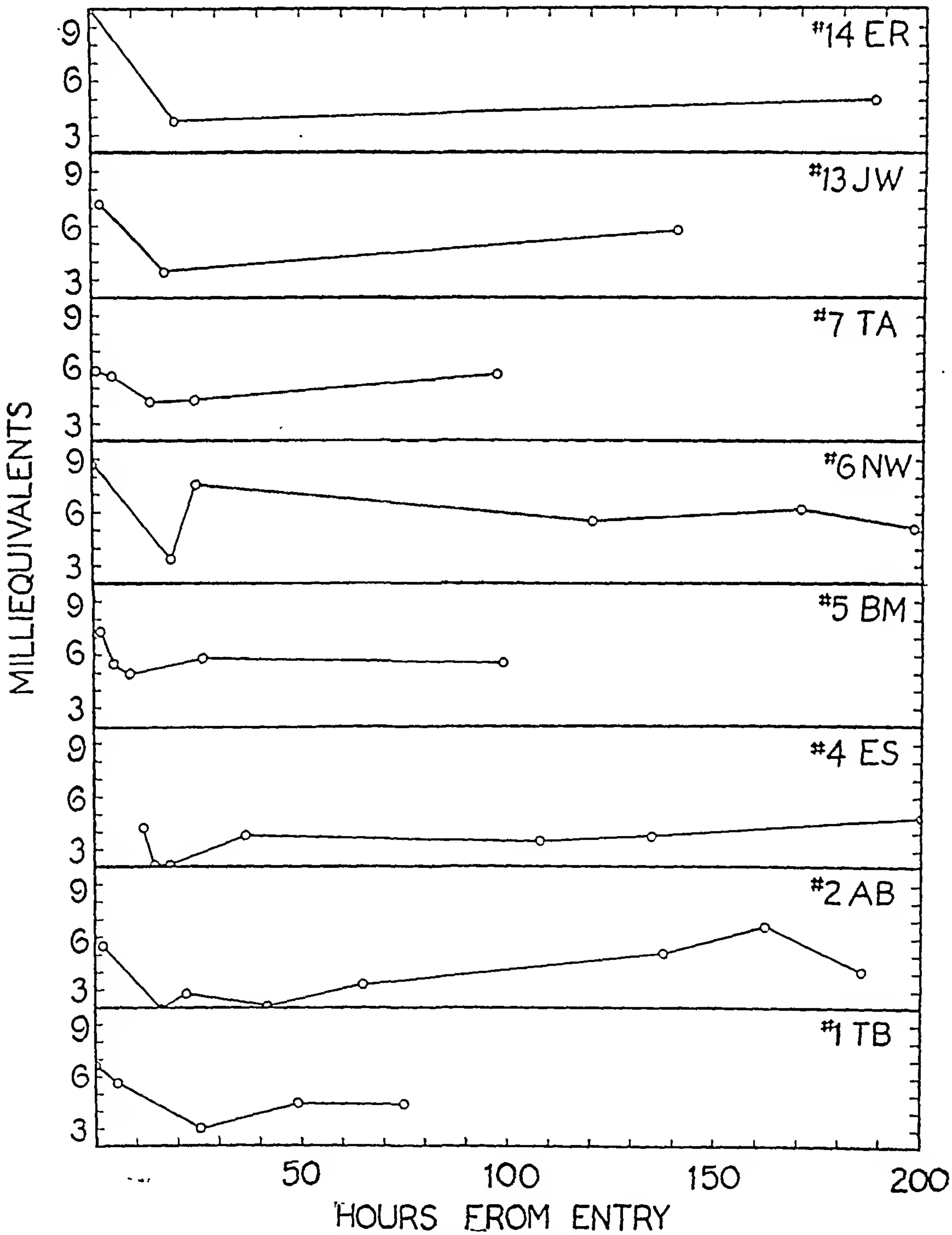

Fig. 1. Serum Potassium Levels During Therapy for Diabetic Acidosis
* Numbers of patients correspond to numbers in Table I.

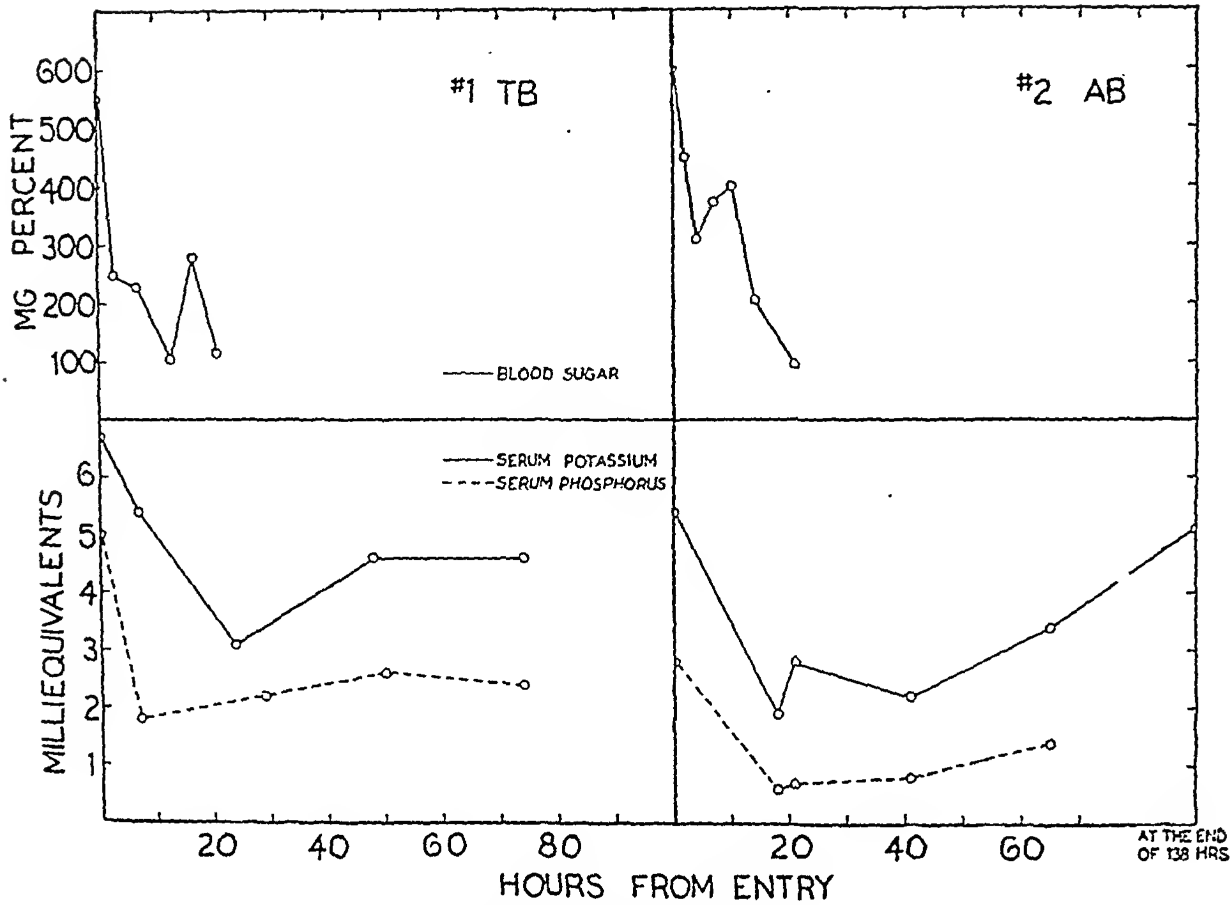

FIG. 2. CORRELATION OF CHANGES IN SERUM POTASSIUM, PHOSPHORUS, AND BLOOD SUGAR LEVELS DURING THERAPY FOR DIABETIC ACIDOSIS

* Numbers of patients correspond to numbers in Table I.

* Phosphorus calculated as $HPO_4^{=}$.

patients had levels under 4 M.eq. Small drops in concentration also occurred with therapy.

6. The wide range of blood sugar level on entry—285 to 1,092 mgm. per cent (5 patients had levels over 800 mgm. per cent), with carbon-dioxide combining power below 9 M.eq. (7 patients had values below 4.5 M.eq.).
7. The wide range of insulin requirement in the first 24 hours—110 to 1,000 units.
8. The large amount of parenteral fluid—4 to 12 liters—given in the first 24 hours, with variation in amount of sodium from 10.5 grams to 27.5 grams, and glucose from 39 to 350 grams.
9. The clinical state varied from coma to consciousness, and 5 of the 14 patients were in shock.

The same general type of electrolyte changes occurred regardless of the amount of insulin, fluids, sodium, or glucose given in the first 24 hours, the rapidity of fall of the blood sugar, or the presence or absence of coma or shock.

More detailed analyses of the changes in the potassium levels are given in Figure 1. This figure demonstrates that the lowest point in potassium concentration usually occurred within the first 24 hours. Not enough serial determinations were made to determine the exact time of the lowest level, and this may vary from patient to patient, but it was usually between 12 and 24 hours. Within 2 to 3 days, the potassium concentrations had returned to normal levels, although in several patients this was delayed for several more days. Potassium concentrations as seen in Figure 1 were usually slightly elevated on entry in the majority of instances. There was no definite correlation

between the rate of fall of the blood sugar, or the total amount of insulin and fluids given in the first 24 hours and the degree of lowering of the serum potassium. However, Figure 2 shows the close correlation in two patients (T. B., No. 1, Table I, and A. B. No. 2, Table I) between fall in blood sugar, inorganic phosphate and potassium levels.

Table II summarizes the range of potassium values found. It is seen that 6 patients (46 per cent) had low potassium values (under 4 M.eq.) and 8 patients had elevated levels (over 6 M.eq.). As it was not always possible to follow some of these patients during the first 24 hours, due to entry on holidays or at night, some abnormalities of potassium levels were doubtless missed.

TABLE II

Range of serum potassium levels in 13 patients

Potassium level	Number of patients*	Per cent of patients
meq.		*per cent*
1–3	2	15
3–4	4	31
Summary of low values	6	46
6–9	7	55
9+	1	8
Summary of high values	8	63

* The same patient may be represented under both low and high levels.

Two of the patients complained spontaneously of marked muscle weakness after therapy. Both showed low serum potassium concentrations at the time.

While the majority of the calcium concentrations fell within the normal range, it was felt that with the fluctuations in pH and albumin and globulin levels the ionized calcium values might show more variation. The relationship between the calculated ionized calcium values and the total calcium concentrations is given in Table III. While the calculated ionized calcium levels tend to follow the fall and rise in total calcium concentrations this is not a constant relationship. This is illustrated by patient B. M. No. 2, Table III. With a total calcium concentration of 5.7 M.eq. the calculated ionized calcium was 2.25 M.eq. or 39 per cent of the total calcium. With a total calcium of 4.4 M.eq. the ionized value was 2.05 M.eq. or 47 per cent of the total.

TABLE III

Relationship between total and calculated ionized calcium levels

Patient	Total calcium	Calculated ionized calcium	Calculated ionized calcium, per cent of total calcium
	meq.	*meq.*	*per cent*
E.S. No. 4*	5.20	2.45	47
	5.00	2.40	48
	5.15	2.30	45
	4.90	2.30	47
	3.55	1.35	38
	4.95	2.05	41
B.M. No. 5*	5.70	2.25	39
	4.40	2.05	47
	4.85	2.10	43
T.B. No. 1*	5.24	2.15	46
	4.64	2.05	44
	4.80	2.20	46
	4.68	2.25	48
	4.98	2.25	45

* Table I.

Magnesium concentrations fell markedly during therapy, particularly during the first 24 hours, although in some instances the maximum fall occurred later. Even after several days, the magnesium concentrations had not returned to the normal level in the majority of the patients. The range of serum magnesium levels is given in Table IV. Forty-one of the 67 determinations (61 per cent) in the 14 patients were below normal, while 11 (16 per cent) were elevated. Five patients showed very low levels—under 1 M.eq.

Correlation between the state of consciousness and the level of magnesium at entrance was found,

TABLE IV

Range of serum magnesium levels in 14 patients

	Magnesium levels	Number of determinations at level indicated	Number of patients(1)
	meq.		
Very low	0.56–1.00	5	5
Low	1.00–1.50	36	13
Normal(2)	1.50–1.92	15	11
Slightly elevated	1.92–2.30	4	3
High	2.30–3.30	7	5

(1) The same patient may be represented under each level due to changes in concentration with therapy.
(2) The normal range for the method used—1.50 to 1.92 milliequivalents.

although none of the values obtained was abnormally high. Three of the patients with magnesium levels over 2.5 M.eq. were in deep coma; one was stuporous. All eight patients with magnesium levels under 2.5 M.eq. on entry were conscious. There was no correlation between the level of magnesium at entrance and the presence or absence of hemoconcentration.

DISCUSSION

Some of the changes in the potassium, calcium, and magnesium levels are undoubtedly due to hemoconcentration or dilution, although study of Table I will show that many of the changes bear no direct relationship to this factor. The concentration of electrolytes at any one moment, regardless of total circulating amount, conditions many of their physiologic functions. This is well attested by the striking changes in the T waves in the electrocardiogram with changing levels of serum potassium (18). It has also been demonstrated that the total circulating amount of any electrolyte, particularly sodium, may be important, as seen in the clinical improvement in patients with Addison's disease after salt therapy, even before the serum sodium concentrations return to normal (19). In general the importance of potassium, calcium, and magnesium in many biologic functions has been adequately reviewed (20, 21, 22).

The marked drop in serum potassium is the most striking of the electrolyte changes noted and has not been stressed previously. The level of 1.9 M.eq. found in one of our patients is, to our knowledge, the lowest level mentioned in a diabetic. Very low levels of serum potassium have been reported in several conditions: sprue—1.1. M.eq. (23); familial periodic paralysis—1.3 M.eq. (24); Addison's disease with therapy—2.9 M.eq. (25).

In diabetes there are several factors relevant to the problem which are known to effect the serum potassium levels. Potassium moves in or out of the cell fairly closely with phosphorus and nitrogen during periods of either tissue catabolism or anabolism (26, 27). With periods of excess tissue breakdown, there is increased urinary excretion of potassium; and, conversely, during protein storage there is decreased urinary excretion of potassium. Administration of certain hormones, or their lack due to disease, can cause such shifts in potassium and nitrogen. Testosterone (28) causes protein anabolism and decreased potassium excretion in the urine, while desoxycorticosterone acetate (19) causes increased urinary excretion of potassium, and decreased extra- and intra-cellular potassium concentrations. Lack of insulin is known to lead to tissue breakdown and a negative nitrogen balance with loss of large amounts of potassium in the urine (5). Administration of insulin leads to a positive nitrogen balance and decreased urinary potassium excretion (5). As the blood levels of potassium and urinary excretion of potassium (and phosphorus) decrease after insulin, it has been suggested (28) that there is an increased intracellular level of potassium and phosphorus, possibly without increase in muscle mass. This possibility must remain a suggestion, however, until determinations of intracellular levels of potassium are made in man following insulin therapy and until the amount which may shift into the liver can be determined. In the normal individual, as well as the diabetic, insulin is known to decrease the serum potassium levels (4, 29, 30). The exact mechanism of this action of insulin on serum potassium is not known, but it has been suggested that it is correlated with shifts of glucose and phosphate into the cell, for formation of a potassium hexosephosphate (see Figure 2), or into the liver in glycogenesis (4, 29, 31, 32).

Another condition that may possibly decrease serum potassium levels in severe diabetic acidosis is the use of large amounts of intravenous saline. Administration of large volumes of fluid parenterally (isotonic) (33) causes loss of some potassium in the urine with the loss of large amounts of sodium. Whether this factor is counterbalanced in diabetics by the effect of insulin in decreasing urinary potassium excretion has not been studied.

Clinically the feature that impressed us as related to the low levels of serum potassium was the marked muscle weakness exhibited by several patients. One of these patients, following a minor insulin reaction, which was treated promptly, showed striking generalized muscle weakness and lethargy. In many respects she resembled the picture seen in familial periodic paralysis. All pa-

tients showed clinical improvement in muscle strength with rise in serum potassium levels. The relationship between muscle weakness, intracellular and extracellular potassium levels is still not settled. Following the use of testosterone, the serum potassium may drop to very low levels, with increased or normal intracellular potassium, and no associated muscle weakness (28). Desoxycorticosterone acetate therapy, if excessive, causes marked loss of potassium, and decreased intracellular potassium concentrations, and is associated with marked muscle weakness (25). In familial periodic paralysis (24) and diabetes with low serum potassium levels there is also marked muscle weakness. The drop in serum potassium concentrations, with decreased urinary excretion during insulin therapy, may be theoretically associated with either increased or decreased intracellular levels, depending on whether the shift is predominantly into the cells, or into the liver in intermediary carbohydrate metabolism and glycogen formation. That there are other factors involved in muscle strength beside intracellular potassium levels has been demonstrated experimentally in rats, who despite very low levels of muscle potassium, were still able to swim (34).

On entry, many of the patients showed elevated levels of potassium. Most of this rise in concentration can be explained on the basis of hemoconcentration. Another factor is decreased renal function secondary to shock which may occur in diabetic acidosis.

The calcium concentrations were maintained at normal or slightly low normal levels in most instances, despite the fact that in diabetic acidosis there is increased urinary excretion of calcium (5). Total circulating calcium is presumably depleted. As many diabetics show marked osteoporosis, periods of negative nitrogen balance and acidosis may be pathogenic factors. The lack of constant correlation between total calcium concentration and percentage ionized fraction is important, as the ionized fraction determines many of the physiologic effects of calcium, such as the length of the electrical systole of the heart. This suggests that both values should be determined.

The mechanism of the marked drop in magnesium concentrations during insulin therapy has not been studied. Possibilities include changes in urinary excretion or use in carbohydrate metabolism with insulin therapy. It should be noted, however, that the amount of magnesium necessary for coenzyme action in the phosphorylation of glucose would appear to be too small to account for the marked changes observed. It is interesting, but not conclusive, that there was some correlation in our series between levels of consciousness and the magnesium concentrations at entry. However, at levels similar to those found in our comatose patients, many individuals appear to have no effects or clinical symptoms. Also, many factors presumably contribute to the state of coma, and no one chemical factor is entirely responsible.

The therapeutic implications of these findings are important. The occurrence of low potassium levels after therapy in 46 per cent of the patients, and very low magnesium levels in 36 per cent of the patients, suggests that these patients should receive potassium and magnesium salts as an adjunct to other therapy. The warning should be given, however, that a diabetic patient in shock, with decreased renal function, should not receive potassium therapy because of the danger of producing dangerously high blood levels (35). This is true also for magnesium. The presumed decrease in total circulating calcium and occasional decreases in the percentage ionized fraction suggest that these salts should be beneficial, too.

SUMMARY

The results of determinations of potassium, magnesium, and calcium levels in 14 patients in severe diabetic acidosis are given.

Forty-six per cent of the patients showed a marked fall in serum potassium levels during therapy. In several patients this was associated with marked muscle weakness. It is suggested that this fall may be related to the effect of insulin on nitrogen storage and urinary excretion of potassium, and the carbohydrate cycle with passage of glucose, phosphorus, and potassium into the muscle cell, or into the liver in glycogen formation.

There was no constant correlation between total calcium concentrations and the ionized fraction. While the concentrations of calcium on entry were usually within the normal range, total circulating amount was presumably decreased, in view of the

hemoconcentration present. This may be a factor in osteoporosis.

There was a marked fall in serum magnesium concentrations in 36 per cent of the patients during therapy, and the levels returned very slowly to the normal range. No explanation can be given for this change. Elevated magnesium levels may play a rôle in the production of coma.

The therapeutic implications of these findings are discussed.

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BIBLIOGRAPHY

1. Hartmann, Alexis F., and Darrow, Dan C., Chemical changes occurring in the body as the result of certain diseases. III. The composition of the plasma in severe diabetic acidosis and the changes taking place during recovery. J. Clin. Invest., 1928, 6, 257.
2. Gamble, J. L., Chemical anatomy, physiology, and pathology of extracellular fluid. Department of Pediatrics, The Harvard Medical School, 1939.
3. Peters, John P., Kydd, David M., Eisenman, Anna J., and Hald, Pauline M., The nature of diabetic acidosis. J. Clin. Invest., 1933, 12, 377.
4. Harrop, George A., Jr., and Benedict, Ethel M., The participation of inorganic substances in carbohydrate metabolism. J. Biol. Chem., 1924, 59, 683.
5. Atchley, Dana W., Loeb, Robert F., Richards, Dickinson, W., Jr., Benedict, Ethel M., and Driscoll, Mary E., On diabetic acidosis. A detailed study of electrolyte balances following the withdrawal and reestablishment of insulin therapy. J. Clin. Invest., 1933, 12, 297.
6. Barber, H. H., and Kolthoff, I. M., A specific reagent for the rapid gravimetric determination of sodium. J. Am. Chem. Soc., 1928, 50, 1625.
7. Kramer, B., and Tisdall, F. F., A clinical method for the quantitative determination of potassium in small amounts of serum. J. Biol. Chem., 1921, 46, 339.
8. Clark, E. P., and Collip, J. B., A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., 1925, 63, 461.
9. Simonsen, D. G., Westover, Leola, and Wertman, Maxine. (In press.)
10. Simonsen, Daisy G., Wertman, Maxine, Westover, Leola M., and Mehl, John W., The determination of serum phosphate by the molybdivanadate method. J. Biol. Chem., 1946, 166, 747.
11. Kingsley, G. R., The determination of serum total protein, albumin, and globulin by the biuret reaction. J. Biol. Chem., 1939, 131, 197.

    Ibid., A rapid method for the separation of serum albumin and globulin. J. Biol. Chem., 1940, 133, 731.

    Ibid., The direct biuret method for the determination of serum proteins as applied to photoelectric and visual colorimetry. J. Lab., and Clin. Med., 1941–2, 27, 840.
12. Van Slyke, Donald D., Stillman, Edgar, and Cullen, Glenn, Studies of acidosis. XIII. Method for titrating the bicarbonate content of the plasma. J. Biol. Chem., 1919, 38, 167.
13. Peters, John P., and Van Slyke, Donald D., Quantitative Clinical Chemistry. The Williams and Wilkins Company, Baltimore, 1932, 11, 527.
14. Benedict, S. R., The estimation of sugar in blood and normal urine. J. Biol. Chem., 1926, 68, 759.
15. Weir, E. G., and Hastings, A. B., The ionization constants of calcium proteinate determined by the solubility of calcium carbonate. J. Biol. Chem., 1936, 114, 397.
16. McLean, Franklin C., and Hastings, A. B., The state of calcium in the fluids of the body. I. The conditions affecting the ionization of calcium. J. Biol. Chem., 1935, 108, 285.
17. Van Slyke, Donald D., Hastings, A. B., Hiller, Alma, and Sendroy, Julius, Jr., Studies of gas and electrolyte equilibria in blood. XIV. The amounts of alkali bound by serum albumin and globulin. J. Biol. Chem., 1928, 79, 769.
18. Martin, Helen Eastman, and Wertman, Maxine, Correlation of the electrocariographic changes in diabetic acidosis with the electrolyte changes. To be published.
19. Thorn, George W., Howard, R. Palmer, and Emerson, Kendall, Jr., Treatment of Addison's disease with desoxycorticosterone acetate, a synthetic adrenal cortical hormone. J. Clin. Invest., 1939, 18, 449.
20. Fenn, W. O., The role of potassium in physiological processes. Physiol. Rev., 1940, 20, 377.
21. Schmidt, Carl L., and Greenberg, David M., Occurrence, transport, and regulation of calcium, magnesium, phosphorus in animal organism. Physiol. Rev., 1935, 15, 297.
22. Darrow, Daniel C., Body-fluid physiology: The relation of tissue composition to problems of water and electrolyte balance. New Eng. J. Med., 1945, 233, 91.
23. Harrison, Harold E., Tompsett, Ralph R., and Barr, David P., The serum potassium in two cases of sprue. Proc. Soc. Exper. Biol. and Med., 1943, 54, 314.
24. Allott, E. N., and McArdle, B., Further observations on familial periodic paralysis. Clin. Sc., 1938, 3, 229.
25. Ferrebee, Joseph W., Ragan, Charles, Atchley, Dana W., and Loeb, Robert F., Desoxycorticosterone esters; certain effects in the treatment of Addison's disease. J. A. M. A., 1939, 113, 1725.

26. Howard, J. E., and Bigham, R. S., Relation of potassium to nitrogen during anabolism and catabolism of protoplasm—Page 7, Conference on Metabolic Aspects of Convalescence, Transactions of Eleventh Meeting, 1945, Massachusetts General Hospital, Boston, Mass.
27. Albright, F., Reifenstein, E. C., Jr., and Forbes, A. P., Does potassium move with nitrogen?—Page 25, Conference on Metabolic Aspects of Convalescence, Transactions of Eleventh Meeting, 1945, Massachusetts General Hospital, Boston, Mass.
28. Butler, Allen M., Talbot, Nathan B., and MacLachlan, E. A., Effect of testosterone therapy on concentration of potassium in serum. Proc. Soc., Exper. Biol. and Med., 1942, 51, 378.
29. Briggs, A. P., Koechig, Irene, Doisy, Edward A., and Weber, Clarence J., Some changes in the composition of blood due to the injection of insulin. J. Biol. Chem., 1924, 58, 72.
30. Clegg, J. L., Serum potassium and serum calcium in insulin shock therapy. Lancet, 1939, 236, 871.
31. Fenn, W. O., The deposition of potassium and phosphate with glycogen in rat livers. J. Biol. Chem., 1938, 128, 297.
32. Cori, C. F., Phosphorylation of glycogen and glucose. Biol. Symposia, 1941, 5, 131.
33. Stewart, John D., and Rourke, G. Margaret, The effects of large intravenous infusions on body fluid. J. Clin. Invest., 1942, 21, 197.
34. Miller, H. C., and Darrow, D. C., Relation of serum and muscle electrolyte, particularly potassium, to voluntary exercise. Am. J. Physiol., 1941, 132, 801.
35. Winkler, Alexander W., Hoff, H. E., and Smith, Paul K., Electrocardiographic changes and concentration of potassium in serum following intravenous injection of potassium chloride. Am. J. Physiol., 1938, 124, 478.

STUDIES ON GANGRENE FOLLOWING COLD INJURY. I. A METHOD FOR PRODUCING GANGRENE BY MEANS OF CONTROLLED INJURY BY COLD[1]

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INTRODUCTION

The incidence of gangrene associated with a wide variety of injuries, in which infection is not a primary factor, continues to present problems of treatment at all levels of handling military casualties from the front line dressing stations to the large general hospitals. Gangrene results from injury due to freezing, prolonged exposure to wet and cold without freezing (immersion or trench foot), burns and vascular occlusion. It seems probable that the fundamental factors responsible for the appearance of gangrene are similar in all of these cases. A more detailed examination than has previously been made of the primary mechanisms by which gangrene is produced, and the steps which may be taken to prevent its occurrence, may have wide application to the general problem of the prevention and treatment of gangrene, irrespective of the primary injury.

Local injury resulting from exposure to cold may take one of several different forms depending chiefly upon the temperature to which the part is exposed. Injury occurring after relatively brief exposure to severe cold, in which the skin and sometimes deeper tissues are actually frozen, will be referred to as "frostbite" (1). It is this type of injury with which this series of papers is primarily concerned. Exposure to milder degrees of cold, especially for long periods of time and under conditions favoring rapid transfer of heat from the body, results in a type of cold injury in which no actual freezing of tissue occurs. This "trench foot" or "immersion foot" type of injury is not specifically considered in this study, although it is recognized that the fundamental features of both may be similar.

The present investigation is an attempt to analyze the factors responsible for the production of gangrene following freezing (frostbite) and to examine methods of treatment of frostbite which are designed to prevent the appearance of gangrene or to reduce its extent. We have not been directly concerned with the surgical management of gangrene following cold injury or with nerve and vascular abnormalities which persist after the acute stages following injury by cold.

SUMMARY OF AVAILABLE PROCEDURES FOR PRODUCING EXPERIMENTAL FROSTBITE

Before it is possible to conduct an adequate study of methods for the treatment of gangrene following experimental frostbite, it is necessary first to obtain reproducible degrees of injury in experimental animals. A variety of methods have been used by previous workers for producing frostbite injury. Since in many cases the results are, to a large degree, dependent upon the method used for producing the frostbite, the available methods will be reviewed briefly.

1. *Liquid immersion.* Some of the first observations on the pathology of frostbite injury, made by Cohnheim (2), were carried out on rabbits' ears frozen by immersion in cold liquids. Lake (3) used liquid immersion in a study of experimental frostbite. Fell and Hanselman (4) produced shock in dogs by freezing the legs in CO_2 and alcohol. Recently Lange and Boyd (5) produced frostbite by liquid immersion, but the limb was first inserted into a boot of thin rubber to protect it from wetting. These authors refer to this procedure as "contact cooling."

2. *Sprays.* In early work Rischpler (6) and others (7) utilized ether spray for producing cold injury. Sprays of ethyl chloride have been used extensively by the Russian investigators Ariev (8, 9, 10) and Orlov (11).

3. *Cold air.* Exposure of a limb to moving cold air most nearly approximates the conditions under which frostbite of face, ears, and hands occurs. Ariev (12) and Ariev and Esberg (9) have reported results obtained by the use of this method. Greene (13) obtained a "stand-

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

ard frostbite" of mouse tails by the use of a small chamber containing the tail, surrounded by a larger chamber filled with solid carbon dioxide.

4. *Direct contact with cold solids.* Precooled metal bars were applied to the skin by Lewis and Love (14) for the production of cold injury. The direct application of solid carbon dioxide to the skin was used by Harkins and Harmon (15) and Tittel (16) and beakers containing solid carbon dioxide were used by Lange and Boyd (5).

The method selected for the production of quantitative frostbite should be one which is applicable to fingers and toes, since injuries in man most often involve these parts (12, 7). Uniform cooling of all surfaces of these parts is difficult by means of the direct application of cold solids. The spraying of volatile liquids upon the skin of appendages is also difficult to control in extent and might be expected to be much more variable in effect than cold air or liquid applied directly. While it is true, as Ariev points out, that cold air is most nearly comparable to the conditions under which frostbite sometimes occurs in man, it does not seem so suitable as liquid immersion for the purposes of producing a quantitative frostbite in experimental animals. The cooling effect of the air is dependent not only upon the temperature, but also upon the air velocity, thus introducing an additional variable. Very long exposures to cold air are necessary to produce frostbite if the velocity is low. Ariev (12) found that after exposing rabbits' ears to still air at a temperature of − 40° C., the ears were still at a temperature of + 11° C. after 4 hours.

The method which we have selected for the production of experimental frostbite involves the immersion of an extremity in a liquid mixture cooled to the desired temperature. Frostbite of rabbits' feet and ears has been compared using varied temperatures from − 10° C. to − 55° C. for a constant time, and using varied times of exposure at − 55° C. Frostbitten extremities in which the injury was produced by exposure to cold air have been compared to those in which the injury was produced by immersion.

Conditions for the production of quantitative frostbite

Rabbits' feet and ears were frozen by immersion in a liquid mixture consisting of 50 per cent by volume ethylene glycol in water, to which were added 150 ml. per liter of 95 per cent ethyl alcohol. This mixture was cooled as needed by the addition of solid carbon dioxide. A mixture of this composition is suitable for cooling to about − 55° C., at which temperature it is still fluid. Lower temperatures may be achieved by the addition of larger amounts of alcohol and CO_2. The relatively high specific heat of this liquid makes it preferable to ether or acetone which have been used by other workers.

Rabbits were anesthetized by the administration intraperitoneally of dial, 90 mgm. per kgm. Occasional experiments were done using pentobarbital (37.5 mgm. per kgm.) anesthesia. The hair was removed from the area to be frostbitten by close clipping (Oster small animal clipper, size 40 blades). Immediately before frostbite the barbiturate anesthesia was supplemented with ether to the point of disappearance of the corneal reflex. This level of anesthesia was found to be necessary to prevent struggling during the period when freezing of the tissue occurred. Hind feet of rabbits were immersed in the freezing mixture as far as the tuberosity at the base of the fifth metatarsal. The distal 4 to 5 cm. of rabbits' ears were frozen in a similar manner. During the period of immersion the ear or foot was kept in motion in order to equalize the temperature of the liquid immediately surrounding it.

Frostbite was produced in a few animals under barbiturate anesthesia only, and in one with local anesthesia (procaine infiltration). The course following injury was not different from that occurring after barbiturate-ether anesthesia.

The ethylene glycol-alcohol mixture was tested for possible irritant properties by immersing rabbits' feet and ears in it at room temperature for periods as long as 30 minutes. These immersions were not followed by hyperemia or by visible alterations of any kind from the time of removal of the part from the mixture to the end of the observation period 3 weeks later.

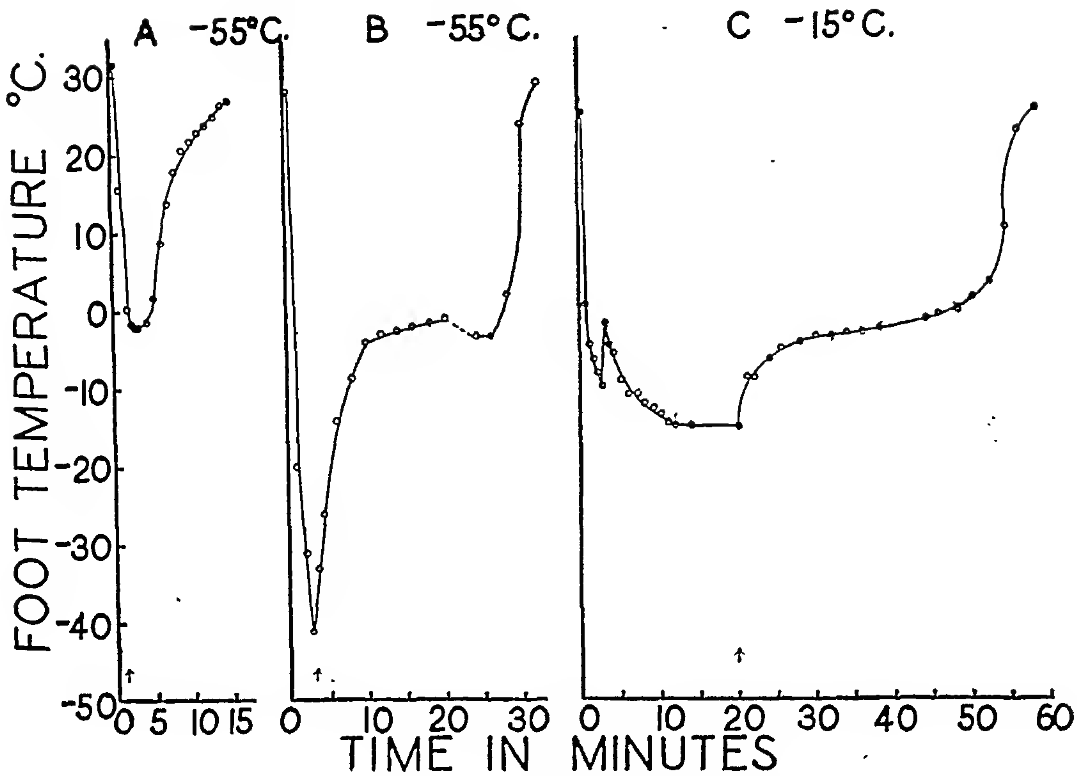
For comparison with the results obtained by immersion in cold liquids, a few rabbits' feet and ears were frostbitten by exposure to a blast of cold air at approximately − 68° C. Compressed air at approximately 18 pounds per square inch pressure was dried, cooled by passing through a chamber filled with solid carbon dioxide, and then directed into a second chamber of about 1 liter capacity which contained the foot or ear to be frozen.

The temperatures of the liquid mixtures and cooled air were measured with Weston bi-metallic thermometers which were checked at intervals by comparison with mercury thermometers.

Deep tissue temperatures in rabbits' feet during immersion in freezing liquid

In order to determine the extent of temperature reduction during frostbite, thermocouples were implanted in the feet of anesthetized rabbits and the temperature determined during immersion in the freezing liquid and during the thawing period after removal from the cold liquid.

A thermocouple made of No. 26 gauge iron-constantan wire was inserted into the foot above the level of immersion and thrust distally deep into the foot at the base of the toes. Temperatures were determined by means of a Leeds and Northrup type K potentiometer. Temperature measurements were made during three different types of frostbite: (a) 1-minute immersion at − 55° C., (b) 3-minute immersion at − 55° C., and (c) 20-minute immersion at − 15° C. The results of representative experiments are given in Figure 1.



DEEP TISSUE TEMPERATURE CHANGES IN RABBITS' FEET DURING FREEZING AND THAWING

Temperature measurements made by means of thermocouples implanted deep in the foot near the base of the toes. See text for complete description. In each case the foot was immersed in the freezing liquid at 0 time and withdrawn at the time indicated by the arrow.

FIG. 1–A. 1-minute immersion at − 55° C.

FIG. 1–B. 3-minute immersion at − 55° C. Thermocouple moved slightly in foot at point indicated by dotted line.

FIG. 1–C. 20-minute immersion at − 15° C. The break in the curve at 3 minutes represents temperature changes resulting from supercooling (see text).

During 1-minute immersion at − 55° C. the deep tissue temperature falls to approximately zero (Figure 1–A). Removal from the liquid is followed by a slight additional fall in temperature to about − 2° C. Upon removal the foot is stiff and the superficial tissues are solidly frozen. The foot remains frozen (with deep temperature at 0° or below) for about 5 minutes, and then warms until it reaches 27° C. after about 15 minutes.

During 3-minute immersion at − 55° C. the lowest temperature reached by the deep tissues was − 40° C. (Figure 1–B). After removal from the freezing mixture the foot warmed slowly until blood flow was reestablished 27 minutes after immersion. Rapid warming of the foot by the blood then raised the temperature to about 30° C. within the next 5 minutes.

During immersion at − 15° C. the foot froze after about 3 minutes and reached temperature equilibrium with the bath in 12 minutes (Figure 1–C). The phenomenon of supercooling, which has been observed frequently in biological material (17), is well illustrated in Figure 1–C. The foot cooled to − 9.4° C. in 155 seconds without freezing. During the next 28 seconds the temperature increased to − 1.5° C. and freezing occurred. The frozen foot then cooled to the temperature of the liquid. After removal from the freezing mixture the foot temperature remained below zero for 28 minutes and then increased rapidly.

EXPERIMENTAL RESULTS

Incidence of gangrene and extent of tissue loss following untreated cold injury

The general course of events leading to gangrene in frostbitten ears and feet will be considered in a subsequent paper. The data given here illustrate the uniformity with which gangrene is produced by controlled cold injury and serve as the basis for evaluation of the methods of treatment to be discussed later.

1. *Frostbite of rabbit ears*

Following immersion of the ear for time periods ranging from 60 seconds to 10 minutes, at temperatures from − 32° C. to − 70° C., the entire frostbitten portion of the ear became gangrenous and was eventually lost. Shorter periods of immersion at − 55° C. led to loss of varying amounts of the distal part of the ear.

In two animals the ears were frostbitten by means of an air blast at a temperature of − 68° C. In one case, after a 2-minute exposure, the ear became edematous, but no loss of tissue occurred. In the other, after 4-minute exposure, complete loss of the injured region resulted.

The data showing the extent of tissue loss together with the time after exposure at which gangrene appeared are given in Table I. The injuries selected for the study of methods of treatment were (a) 1½-minute immersion at − 55° C., and (b) 1-minute immersion at − 55° C. The extent of tissue loss and the extent and rate of development of gangrene following these injuries were quite uniform, as may be seen from Table I. In every instance the whole of the distal portion of the ear to the point of immersion became gangrenous and separation occurred at the line to which the ear had been immersed. Following 1½-minute exposure at − 55° C., wet gangrene appeared in about 70 per cent of the animals on the third day after immersion and drying occurred on the fifth day. Following 1-minute exposure at − 55° C., 80 per cent of the animals developed wet gangrene on the third day and dry gangrene on the fifth day. The time of separation of the injured part of the ear varied somewhat depending upon the activity of the animal in the cage.

TABLE I

Incidence of gangrene and extent of tissue loss following frostbite of rabbit ears

Duration of immersion	Temp.	No. of animals	Per cent of animals developing gangrene on days after injury: Wet stage, 3 days	Wet stage, 4 days	Dry stage, 4 days	Dry stage, 5 days	Dry stage, 6 days	Extent of tissue loss
A. Liquid immersion								
min.	*° C.*		*per cent*		*per cent*			
10	−32	1	—	—	—	—	—	Complete[1]
3	−40	1	—	—	—	—	—	Complete
2	−42	2	—	—	—	—	—	Complete
2	−53	2	100	0	0	100	0	Complete
1½	−55	24	71	29	17	72	11	Complete
1	−55	11	80	20	20	80	0	Complete
1	−70	1	—	—	—	—	—	Complete
½	−55	1	0	100	0	100	0	80 per cent of injured region
¼	−55	1	100	0	0	100	0	80 per cent of injured region
B. Air blast								
2	−68	1	No gangrene					None
4	−68	1	100	0	0	0	100	Complete

[1] Complete loss indicates loss of entire injured part of ear as far proximally as the line to which it was immersed.

TABLE II

Extent of tissue loss occurring after frostbite of rabbit feet

Duration of immersion	Temp.	Number of animals	Extent of tissue loss
min.	*° C.*		
Series 1			
1	−55	12	No tissue loss (11).[1] Distal phalanx of each toe (1).
2	−55	5	All toes (4). Complete except plantar pad (1).
3	−55	16	Complete to line of immersion (11). Complete except plantar pad (4).
Series 2			
3	−45	2	Complete to line of immersion (2).
3	−35	2	Complete except plantar pad (2).
3	−25	2	Distal and middle phalanges of each toe (2)
3	−15	2	Foot did not freeze: no tissue loss (2).
Series 3			
15	−15	1	No tissue loss (1).
30	−15	1	No tissue loss; toe pads thickened (1).
60	−15	5	Complete to line of immersion (2). Complete except plantar pad (2). No tissue loss: foot did not freeze (1).

[1] Numbers in parentheses show number of animals.

2. Frostbite of rabbit feet

The extent of tissue loss and the incidence of gangrene of rabbit feet following frostbite were determined in three series of experiments (Tables II and III).

TABLE III

Development of gangrene following frostbite of rabbits' feet

Duration of immersion	Temp.	No. of animals	Per cent of animals developing gangrene on days after injury					
			Wet stage		Dry stage			
			2 days	3 days	4 days	5 days	6 days	7 days
min.	°C		*per cent*		*per cent*			
1	−55	12	None		None			
2	−55	5	100	0	40	60	0	0
3	−55	15	60	40	20	60	13	7
3	−45	2	100	0	0	50	50	0
3	−35	2	50	50	0	50	0	50
60	−15	4	0	100	0	0	25	75

Series 1 consisted of animals in which one hind foot was immersed for 1, 2 or 3 minutes at a temperature of − 55° C. Following immersion for *1 minute* no loss of tissue occurred in 92 per cent of the animals, and gangrene did not develop in spite of massive edema. After 4 to 7 days the only consequences of the frostbite were thickening of the toe pads and slight to moderate induration of the foot. Following immersion for *2 minutes* gangrene appeared on the toes after the times given in Table III. Spontaneous amputation of the toes took place at varying times after the initial injury. The foot showed some scarring on the dorsum near the base of the toes and sometimes along the edges of the foot extending 0.5 to 1 cm. proximally. The stump was thickened, especially at the distal margin. Immersion at − 55° C. for *3 minutes* resulted in complete loss of the foot as far proximally as the line to which it was immersed in 69 per cent of the cases, and loss of the entire foot with the exception of a narrow tongue of tissue on the plantar surface in 25 per cent of the animals. The incidence of gangrene is given in Table III. Separation near the line of immersion occurred spontaneously; in some cases separation occurred 1 to 2 cm. distal to the line of immersion leaving a dry stump which was then worn away by the movements of the animal.

Series 2 consisted of animals in which one hind foot was immersed for a constant time (3 minutes) at − 45°, − 35°, − 25° and − 15° C. The extent of tissue loss is given in Table II and the incidence of gangrene in Table III. Freezing of the foot did not occur at − 15° C. in 3 minutes. At the other temperatures gangrene developed and varying amounts of tissue were lost, the injury being more severe at the lower temperature.

Series 3 consisted of animals in which one hind foot was immersed for longer periods of time, up to 1 hour, at − 15° C. Freezing of the foot usually occurred after 10 to 15 minutes at this temperature. In one animal, given a supplementary dose of pentobarbital immediately before frostbite and requiring artificial respiration, the foot did not freeze during 1 hour at − 15° C. When freezing did not occur edema was absent and gangrene did not develop. Following immersion of 60 minutes at − 15° C. the final tissue loss involved the entire foot in 2 cases and all except a narrow segment of the plantar surface in two others. The foot retained a flattened shape, with toes spread, for several weeks, and induration was severe. Gangrene developed somewhat later in these animals than in those injured at lower temperatures (Table III).

The animals comprising Series 1 and those in Series 3 injured by immersion for 60 minutes at − 15° C. serve as controls for evaluation of methods of treatment of experimental cold injury to be considered in a subsequent paper.

Effect of precooling on the extent of tissue loss following frostbite of rabbit feet

In three animals the foot was immersed in the liquid mixture at a temperature of 0° to + 2° C. for 30 minutes preceding a frostbite of 1-minute duration at − 55° C. In all instances the resulting injury was more severe than following a frostbite of only 1 minute at − 55° C. Parts of the toes of all three animals were lost; in one the loss involved one toe and the distal phalanx of another; in the second, only the distal phalanx of one toe; and in the third, the distal and middle phalanges of two toes. In contrast to these results, frostbite of 1 minute at − 55° C. without precooling usually resulted in no loss of tissues, but only thickening of the toe pads (Table II).

One animal was placed in a cold room at + 4° C. for 2 days preceding frostbite. The hair had been removed from both feet. Exposure of one foot for 1 minute at − 55° C. immediately upon removal from the cold room resulted only in the usual thickening of toe pads and no loss of tissue. The rectal temperature upon removal from the cold room was 38.7° C.

On one animal the hair was removed from both feet and the animal was placed in the cold room at + 4° C. for 24 hours. The animal was then anesthetized with dial and returned to the cold room for 2 hours, by which time the rectal temperature had fallen to 35° C. Following frostbite for 1 minute at − 55° C. the foot was slightly thickened, but no loss of tissue ensued.

SUMMARY

Controlled cold injury (frostbite) of rabbit ears and feet was produced by immersion of the depilated part in a water-ethylene glycol-alcohol mixture cooled to the desired temperature by means of the addition of solid carbon dioxide.

The immersion of ears of anesthetized rabbits in liquid at temperatures ranging from − 32° C. to − 70° C. for periods of 1 to 10 minutes resulted in freezing of the ear, edema, gangrene and spontaneous amputation of the entire injured part of the ear. Immersion for 15 or 30 seconds at − 55° C. resulted in loss of about 80 per cent of the injured part of the ear.

The effects of immersion of the hind feet of anesthetized rabbits in cold liquid varying from − 15° C. to − 55° C. for different lengths of time were determined. At − 55° C. the extent of tissue loss could be increased by increasing the duration of exposure from 1 to 4 minutes. At − 15° C. the deep tissue temperature of a foot became equal to that of the bath in 15 minutes. At this temperature the extent of tissue loss could also be increased by increasing the duration of exposure. When the time of exposure was maintained constant at 3 minutes and the bath temperature varied from − 15° C. to − 45° C., progressively greater loss of tissue was encountered with reduction of exposure temperature.

Precooling of feet for 30 minutes at about + 2° C. increased the severity of injury following a subsequent exposure for 1 minute at − 55° C. Gangrene did not result from exposure for 1 hour at − 15° C. if freezing of the tissue did not occur. Gangrene resulting from cold injury was qualitatively identical in each case regardless of the time or temperature of exposure.

Readily reproducible degrees of injury and amounts of tissue loss could be obtained by exposure at a given temperature for a given period of time.

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BIBLIOGRAPHY

1. British Medical Research Council, A guide to the preservation of life at sea after shipwreck. War Memo. No. 8 (1943). Brit. Med. Bull., 1944, 2, 150.
2. Cohnheim, J., Neue Untersuchungen über die Enzündung. Berlin, 1873.
3. Lake, N. C., Report upon an investigation into the effects of cold upon the body. Lancet, 1917, 2, 557.
4. Fell, E. H., and Hanselman, R., Prevention of shock and death by immediate application of pressure dressing to severely frozen limbs of dogs. Ann. Surg., 1943, 117, 686.
5. Lange, K., and Boyd, L. J., The functional pathology of experimental frostbite and the prevention of subsequent gangrene. Surg., Gynec. and Obstet., 1945, 80, 346.
6. Rischpler, A., Ueber die histologischen Veränderungen nach der Erfrierung. Beitr. path. Anat., 1900, 28, 541.
7. Sonnenburg, E., and Tschmarke, P., Die Verbrennungen und die Erfreierungen. Neue Deutsche Chirurgie, 1915, 17.
8. Ariev, T. Y., Experimental observations on the brittleness of frozen extremities. Vestn. Khirur., 1937, 52, 63.
9. Ariev, T. Y., and Esberg, N. A., Blood changes in frostbite. Vestn. Khirur., 1937, 51, 19.
10. Ariev, T. Y., Slow vs. rapid warming of frozen extremities. Vestn. Khiur., 1939, 57, 527.
11. Orlov, G. A., On the influence of novocaine block on the healing of tissues exposed to cold. Khirurgia (Moscow), 1937, No. 10, p. 15.
12. Ariev, T. Y., New findings in pathology and clinical behavior of frostbite. Sov. Vrach. Gaz., 1939, No. 7, 391.

13. Greene, R., The immediate vascular changes in true frostbite. J. Path. & Bact., 1943, 55, 259.
14. Lewis, T., and Love, W. S., Vascular reactions of the skin to injury. III. Some effects of freezing, of cooling and of warming. Heart, 1926, 13, 27.
15. Harkins, H. N., and Harmon, P. H., Thermal injuries: the effects of freezing. J. Clin. Invest., 1937, 16, 213.
16. Tittel, S., Ueber die Reaktionsweise des Gefässsystems bei lokaler Erfreierung. I. Die Art des Reaktionsablaufes an den einzelnen Abschnitten des Gefasssystems. Ztschr. ges. exper. Med., 1943, 113, 698.
17. Bělehrádek, J., Temperature and living matter. Protoplasma Monographien, 1935, 8, 117.

STUDIES ON GANGRENE FOLLOWING COLD INJURY. II. GENERAL COURSE OF EVENTS IN RABBIT FEET AND EARS FOLLOWING UNTREATED COLD INJURY[1]

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In part I of this series (1) a method for the production of controlled cold injury (frostbite) of the ears and feet of rabbits was described, and data were given illustrating the incidence of gangrene and extent of tissue loss following injury. The general course of events, including gross changes, skin color, and temperature measurements of injured ears and feet will be given here.

EXPERIMENTAL RESULTS

A. Gross changes following injury

Following immersion of the ear of rabbits for periods ranging from 60 seconds to 10 minutes, at temperatures from − 32° C. to − 70° C., the entire frostbitten portion of the ear becomes gangrenous and is eventually lost (1). Loss of the entire injured part of hind feet occurs after exposure for 3 minutes at temperatures of − 45° C. or lower and after 60 minute exposure at − 15° C. The gross changes following injury of various degrees in which actual freezing of the tissues occurs are at first similar enough so that a general description will suffice.

Immediately upon removal from the freezing mixture the ear or foot is solidly frozen, stiff and pale greyish-pink in color (Figure 1–A). The ear and toes are not "brittle" in that they cannot be broken when bent between the fingers. This observation is in agreement with the work of Ariev (2) who found that brittleness of frozen fingers, ears, and whole frogs and mice did not occur until a tissue temperature of about − 80° C. was reached.

In ears, thawing is first evident in the region immediately surrounding the central artery and in the proximal frostbitten region immediately distal to the unfrozen part of the ear. After about 3 minutes, blood flow in the larger vessels becomes apparent. Within 5 minutes, the frozen region is thawed. The vessels are engorged with blood and the temperature of the frostbitten portion of the ear rises rapidly. Swelling of the ear is first evident in the region immediately surrounding the larger vessels, especially around the central artery. The edema first involves only the frostbitten region and then extends proximally until the entire ear is swollen to approximately 4 times the initial volume (3). During the first several hours after injury the advancing edge of the edematous area is clearly visible and may be seen to progress proximally toward the base of the ear. This edge is frequently pointed in the center, with the point lying in the region of the central artery. Blisters form on the inner surface of the frostbitten portion of the ear about 1 to 6 hours after frostbite. They are most commonly seen overlying the central artery, about 1 cm. from the tip. Profuse exudation of fluid from the injured portion of the ear is seen 1 to 2 days after frostbite. A crust of dried protein usually forms.

Three to 4 days after injury the frostbitten portion of the ear becomes cool and the cartilage of this region appears to be entirely lacking in elasticity. This change marks the beginning of the period of "wet gangrene" during which no blood flow is demonstrable. Drying becomes grossly evident by the fourth to the sixth day. Fluid loss from the gangrenous tissue occurs largely by surface evaporation, although in the earlier stages some may be taken up via the lymphatics and intact circulation of the neighboring normal tissue. The dried mummified part may remain attached for several weeks but is eventually lost. Loss of tissue has in all cases involved only the frostbitten area and does not extend into the proximal, un-

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

injured portion of the ear. The edge of the surviving stump is at first somewhat red and thickened by induration. The redness and induration disappear within a few weeks and are replaced by firm scar tissue well covered by hair-free epithelium at the edge where separation occurred.

In feet, thawing begins at the proximal part of the frostbitten region. It occurs first superficially, and the skin may become pliable while the deeper tissues are still hard. Once the foot is thawed it warms rapidly to temperatures about 5° C. below body temperature. Swelling begins when blood flow is reestablished. It occurs first only in the injured region, and then extends up the leg (Fig. 1–B). The color of the foot becomes darker within 2 hours; at this and somewhat later times the dorsal surface of the foot may be engorged with blood, extremely cyanotic, and moist. Exudation occurs through any skin abrasions. Swelling reaches a maximum in the frostbitten region within 6 hours after injury, although further fluid loss to the foot may occur after this time (3). Since the skin over the frostbitten part of the foot is maximally distended, the fluid is forced into the leg and may reach the thigh. After 1 to 4 hours, blisters appear on the hair-free toe pads and are always confined to that region. The edema begins to diminish after 1 to 3 days, depending upon the severity of the injury. Exudation and wet gangrene follow severe injuries (1). Fluid is lost both externally and internally as in the ears. The foot shrinks and areas of wet gangrene become dry within 2 to 3 days (Figure 1–C). The dried, mummified part remains attached for several weeks (Figure 1–D). In a few cases (about 2 per cent, all involving colored rabbits) the animals chewed the dead portions and rapidly removed the stump. More often it was worn away by the movement of the animals in the cage or separated spontaneously after about 3 weeks.

B. Skin color changes following injury

In spite of the ultimate development of gangrene in frostbitten ears and feet of rabbits there is abundant evidence that, for some period between the moment of injury by cold and the appearance of unmistakable signs of ischemia and death of tissue, blood flows through the injured region, often at faster rates than are encountered in uninjured tissues.

In the period immediately after the frozen part is removed from the freezing mixture and before the completion of thawing, the skin color changes from a faintly pink, opaque grey to bright pink. The bright pink color appears first over the large blood vessels and is most strikingly apparent in the ear. The color change in the ear is noticeable within the first 3 minutes after removal from the freezing mixture. Within 5 minutes, the pink color deepens to a bright red and spreads in all directions from the larger vessels. Edema formation begins early in this period of hyperemia, but, in spite of the accumulating fluid, the injured part does not begin to take on a cyanotic appearance until the edema is well advanced. In sharp contrast to the persistent bright red color of the injured regions when they are allowed to thaw at room temperature is the immediate deep cyanosis that is produced by rapid thawing of the tissues in water at 41° to 42° C. (a method of treatment to be described later). In the rapidly thawed ears and feet, good arterial pulses can be felt, but cyanosis is present and marked from the time of return of blood flow.

Examination of the central artery of the ear in the region proximal to the injured portion shows that the period of marked dilatation lasts for about 1 hour after frostbite. Between the first and second hour after frostbite, there is a progressive constriction of the central artery in the normal part of the ear while the portion of the artery within the injured area retains its wide diameter. Close observation with the aid of transillumination shows that the apparent gross diameter of this vessel does not correspond with the width of the column of blood contained but is, in part, due to perivascular edema.

In general, skin color of the normal part of the ear parallels accurately the changes in blood flow which are indicated by the skin temperature measurements described below. Between 4 and 24 hours after frostbite, the massive edema and hyperemia tend to obscure the early sharp differentiation between the appearance of the frostbitten and the normal part, but demarcation of the two regions again becomes clear with the development of deepening cyanosis of the injured area. The

association of the cyanosis with reduced flow of blood is indicated by the accompanying fall in skin temperature of the distal portion of the ear as compared with the uninjured portion and by the weakening and final disappearance of palpable pulsations in the distal segment of the central artery.

While the details of the circulation cannot be so clearly made out in the frostbitten feet of rabbits, the changes in skin color are very similar to those observed in the ears.

C. *Skin temperature changes following cold injury*

1. *Methods for measurement of skin temperature*

Measurements of skin temperature of rabbit feet and ears following frostbite were made continuously during the first day following injury and at intervals during the next 120 hours. Readings were taken routinely for 5 minutes with each of 5 iron-constantan thermocouples which were soldered to thin copper discs 7 mm. in diameter and secured to the skin by means of "Sealskin" (polyvinyl butyral in isopropyl alcohol and castor oil). Temperatures were recorded on a Leeds and Northrup Micromax recording potentiometer.

On frostbitten feet the thermocouples were placed between the third and fourth toes and on the dorsal and plantar surfaces of the foot in the midline, about 1.5 cm. proximal to the base of the toes. On the normal foot the thermocouples were placed in the same relative positions, although in many cases only the toe and dorsum temperatures of the normal foot were measured.

On frostbitten ears one thermocouple was placed about 1 cm. from the margin and far enough laterally from the midline to avoid the central artery, another about 2 to 3 cm. proximal to this in the injured region, and a third about 3 cm. proximal to the line to which the ear was immersed.

Although the measurements of skin temperature were not made in a constant temperature room, the air temperature of the laboratory was relatively constant (25° C.) and varied only about ± 1° C. from day to day and throughout a single day. Care was taken to shield the thermocouples from ambient air currents by use of a tubular gauze sock slipped over the ear or foot after the thermocouples were in place. Some experiments were carried out with the foot or ear encased in a cellophane jacket. It was found that the skin temperature changes were very similar whether or not the cellophane jackets were used, except that gangrenous toes and tips of ears tended to be warmed by the warm air inside the cellophane. The experiments reported are those in which cellophane was not used.

2. *Time course of temperature changes following injury*

Following 60- or 90-second exposure of an ear at − 55° C., the temperature of the frostbitten portion increases rapidly until the maximum is reached in 20 to 60 minutes. This temperature is usually 32° C. to 35.5° C. The proximal, uninjured part of the frostbitten ear is usually several degrees warmer than that of the contralateral ear. The temperature of the frostbitten part of the ear is maintained within a few degrees of the maximum temperature attained for the first 3 to 5 hours following injury; after this a progressive fall in skin temperature is observed. Figure 2 shows the mean skin temperature of the ears of 3 animals for 120 hours following 90-second immersion at − 55° C. It is clear from this figure that the mean temperature of the tip of the injured ears is lower than that of the normal ears 5 hours after injury and that it falls continuously during the first 50 hours after injury until it reaches a level 1° to 2° C. above room temperature. Wet gangrene appears about 48 hours after injury and drying occurs after 72 hours. Thus the reduction in blood flow to the ear is not immediately brought about by frostbite but is a continuous process occurring during the first 50 hours after exposure. The stabilization of skin temperature of the injured region at a low level marks the beginning of the stage of wet gangrene.

FIG. 1. GENERAL COURSE OF EVENTS FOLLOWING FROSTBITE OF RABBIT FEET PRODUCED BY LIQUID IMMERSION

A. Appearance of frozen foot, 20 minutes after removal from freezing mixture at − 52° C. (3 minute immersion). Thawing has not yet occurred.

B. Edematous foot 3 hours after immersion for 3 minutes at − 45° C. Foot volume 2.6 times initial volume.

C. Appearance of foot 4 days after injury produced by immersion for 3 minutes at − 55° C. showing wet gangrene on the dorsal surface.

D. Dry, mummified foot 7 days after frostbite produced by immersion for 3 minutes at − 55° C.

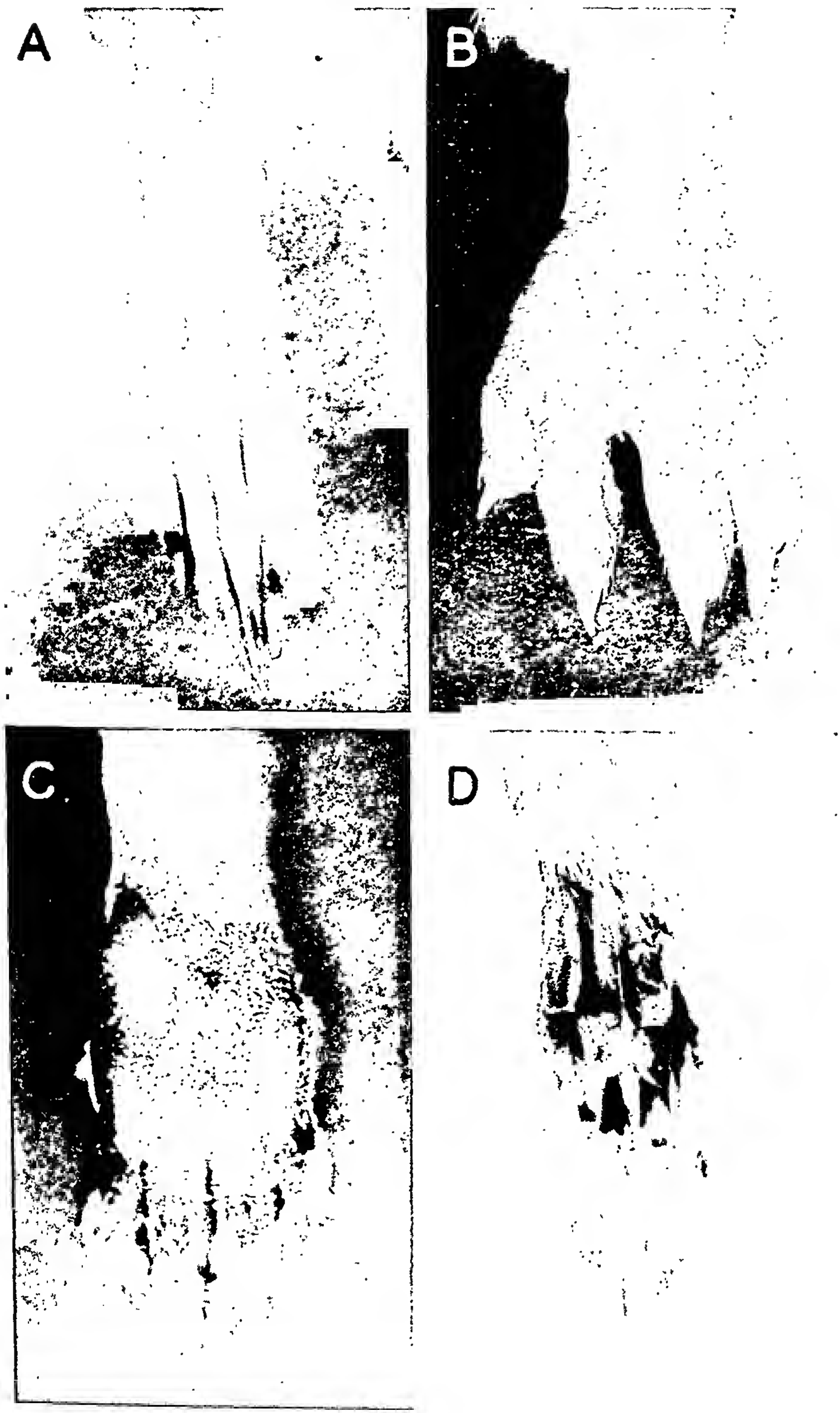

FIG. 1

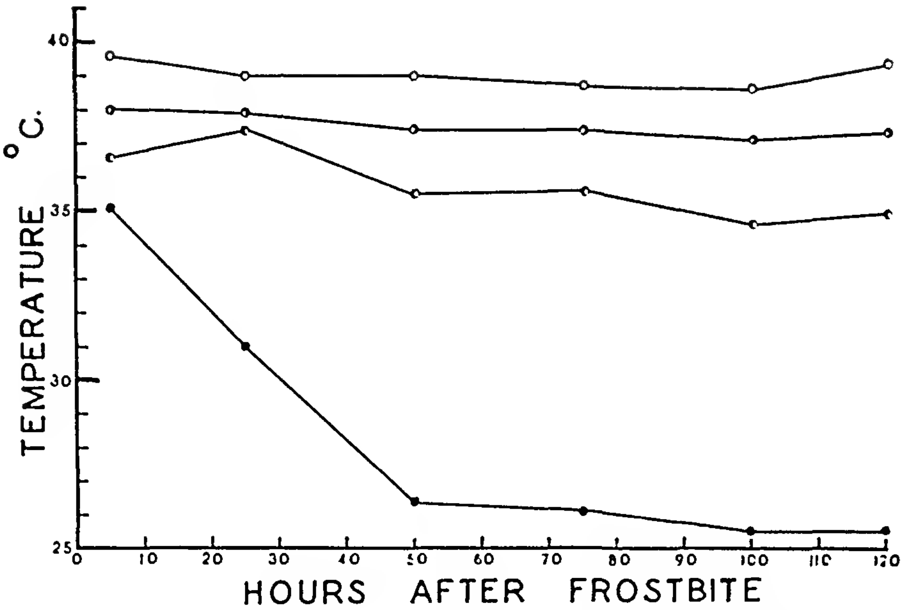

FIG. 2. TIME COURSE OF SKIN TEMPERATURE CHANGES IN RABBIT EARS AFTER EXPOSURE OF THE DISTAL PART OF THE EARS AT − 55° C. FOR 90 SECONDS. RECTAL TEMPERATURES ARE ALSO SHOWN

Mean data from three animals are plotted in the figure.

Open circles........Rectal temperature
◗ ....Unexposed proximal portion of frostbitten ear
◐ ....Tip of normal ear
Solid circles........Tip of frostbitten ear

Rabbits' feet removed from the freezing liquid remain solidly frozen for about 20 minutes after 3-minute immersion at − 55° C. After thawing, the temperature rises rapidly as blood flow to the injured region is reestablished. Within 1 hour the temperature of the frostbitten toes reaches a maximum of 28° to 31° C. This temperature is maintained for a variable period, sometimes not more than 20 to 30 minutes; a progressive fall in temperature of the toes then occurs until it reaches 26° to 30° C. after 4 hours. The maximum temperature of the dorsum of the injured foot is attained 20 to 30 minutes earlier than that of the toes and remains 2° to 3° above that of the toes. After 4 hours it has fallen to 31° to 33° C. Figure 3 shows the mean temperatures of frostbitten feet (3 minutes exposure at − 55° C.) and the contralateral feet of three animals during a period of 120 hours after injury. It may be seen that the dorsum and toes of the frostbitten feet show consistently lower temperatures than does the dorsum of the normal foot for the first 50 hours after injury. After this time the skin temperatures of the dorsal surfaces of the injured and normal feet closely parallel each other. The temperature of the frostbitten toes remains lowest during the entire time and eventually falls even farther when the toes dry and become mummified. The tissue loss in these animals (Figure 3) involves the toes and most of the dorsal surface of the feet. The relatively high temperature of the dorsum of the injured feet may be attributed to persistence of blood flow in deeper vessels, arising from the plantar arch. A limited number of measurements of skin temperature of the plantar surface of the foot indicates that it may be several degrees higher than that of the dorsal surface.

When the foot is exposed at − 55° C. for only 1 minute, which usually produces only severe edema but no loss of tissue, the initial temperatures which are attained by the toes of the frostbitten foot are higher than after 3 minutes of exposure. Within 1 hour after injury the toes usually reach a temperature of about 35° C. (33° to 38° C.), and then fall several degrees during the first 4 hours. The temperature of the dorsum of the frostbitten foot is often 1° to 2° C. higher than that of the contralateral dorsum. In 2 animals, in which the toes were lost following a 1-minute exposure at − 55° C., the temperature of the toes

dropped sharply after 40 to 50 hours to a level only 2° to 3° C. above room temperature. This drop in temperature of the toes, which are eventually lost, is progressive and coincides in time with the other local signs of gangrene.

3. *Relationship of skin temperature changes to tissue loss*

The temperatures of normal and frostbitten feet have been further analyzed in consideration of the extent of subsequent tissue loss in the injured feet. The temperature of the toes of the frostbitten foot has been compared with the temperature of the dorsal surface of the normal foot of the same animal at various times during the first 6 hours after injury. The data for these comparisons are given in Table I.

The column in Table I headed "Temperature difference" gives for each animal the mean of a number of comparisons between the temperatures of the injured toes and the dorsum of the normal foot during the first 6 hours. The column headed "P" expresses the probability that such differences would occur through errors in random sampling. These probabilities were calculated by the "t" test of Fisher (4).

TABLE I

Comparison of skin temperatures during the first six hours after injury in normal and frostbitten rabbit feet
Feet frostbitten by immersion at −55° C. for the time indicated

Duration	Tissue loss	Skin temperature. Mean of determinations during first six hours		Temperature diff. ° C.	P[1]
		Frostbitten	Normal	FB–N	
1	None	36.8°	32.8°	+4.0°	0.500
1	None	34.1°	36.0°	−1.9°	0.020
1	None	38.5°	38.5°	0	0.200
1	None	37.9°	35.8°	+2.1°	0.020
1	Distal phalanges	33.6°	35.6°	−2.0°	0.010
1	All digits	29.8°	37.0°	−7.2°	0.001
3	Entire foot	27.5°	30.9°	−3.4°	0.001
3	Entire foot	29.7°	35.8°	−6.1°	0.001
3	Entire foot	32.8°	35.7°	−2.9°	0.001

[1] *P* expresses the probability that such differences would occur through errors in random sampling.

At a time after injury when the gross appearance of the injured feet provides no indication of impending gangrene, differences in skin tempera-

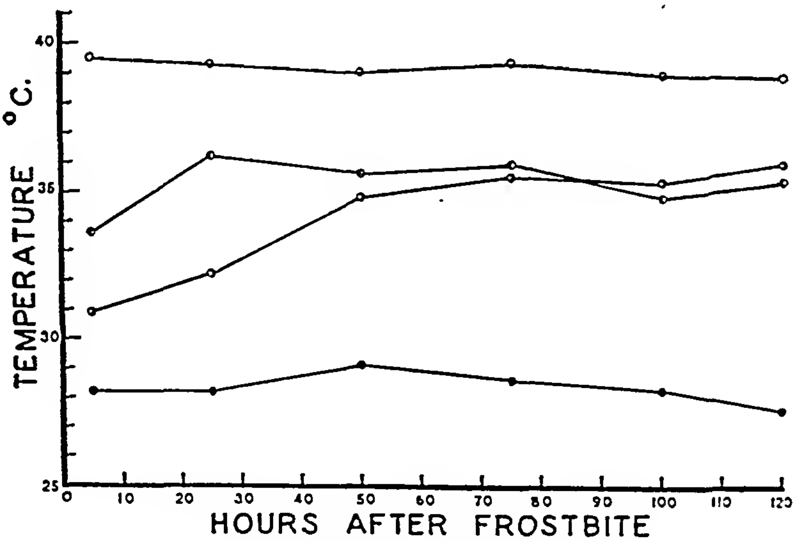

Fig. 3. Time Course of Skin Temperature Changes in Rabbit Feet after Exposure of Feet at −55° C. for Three Minutes. Rectal Temperatures Are also Shown

Mean data from four animals are plotted in the figure.

Open circles........Rectal temperature
........Dorsum of normal foot
........Dorsum of frostbitten foot
Solid circles........Toes of frostbitten foot

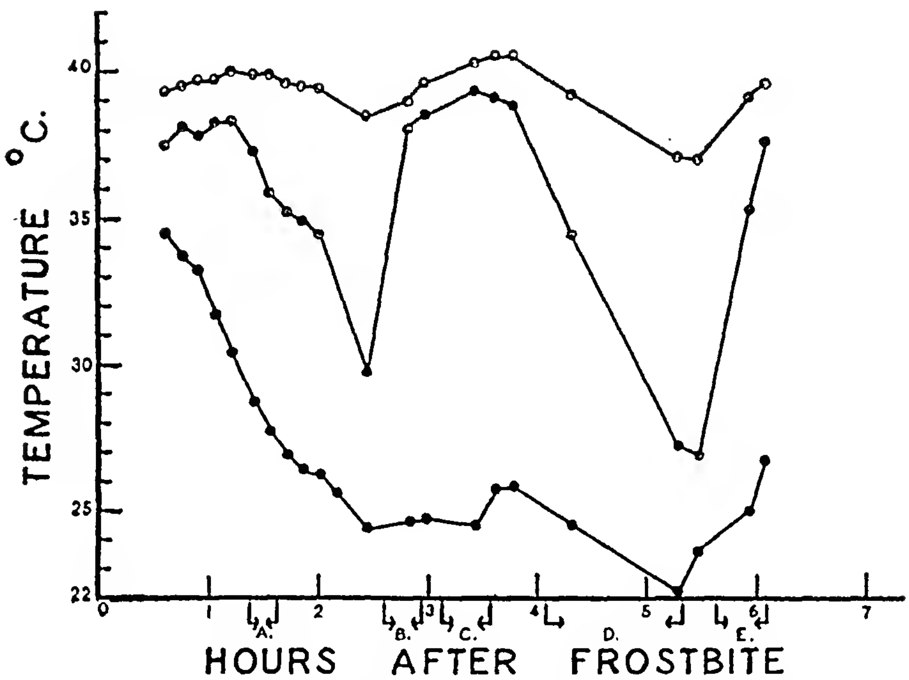

FIG. 4. SKIN TEMPERATURE CHANGES IN NORMAL AND FROSTBITTEN RABBIT FEET REPRESENTING REFLEX RESPONSES TO GENERAL HEATING AND COOLING

Representative data from one animal. Foot immersed at − 55° C. for 3 minutes.

Open circles.........rectal temperature
Cross in circle.......toes of normal foot
Solid circles.........toes of frostbitten foot

A—Ears cooled with ice.
B—Ears warmed.
C—Ears and body warmed.
D—Body cooled with wet towels, ice, and fan.
E—Body warmed with heating pads.

ture were found to be of decided value in predicting the ultimate loss of tissue. The data in the table illustrate the association of low skin temperature of the injured parts and eventual development of gangrene. Following 1-minute exposure at − 55° C., in the animals in which no tissue loss resulted, the mean temperature differences between the normal and injured feet ranged from + 4.0° to − 1.9° C. and the probability that such differences would occur through errors in random sampling ranged from 2 in 100 to 50 in 100 ($P = 0.02$ to 0.5). For comparison with these are 2 animals which lost parts of the toes following exposure for 1 minute at − 55° C. In both of these animals the temperature of the toes of the injured foot was consistently lower than that of the normal foot. The smaller values of P (< 0.01 and 0.001 respectively) indicate that the differences are statistically significant. Following 3-minute exposure at − 55° C., the temperature of the injured feet was always lower than that of the normal feet and these differences are also statistically significant ($P < 0.001$). From the data given in Table I it is apparent that as early as the first 6 hours after injury the temperature of the toes of the injured foot is significantly lower than that of the normal foot in those animals in which the toes are subsequently lost. As described previously this temperature difference becomes more marked with time, so that with the appearance of wet gangrene the temperature of the gangrenous part is only slightly above that of the environment.

4. *Vascular spasm in extremities during the first hours after injury*

Temperature changes in frostbitten and normal extremities of rabbits have been studied during

the application of heat and cold generally as well as locally to a part remote from that injured by frostbite. In all cases the experiments were carried out during the 7-hour period immediately following frostbite, at a time when the animals were still anesthetized with dial. The dial was given, in most cases, approximately 1 hour before frostbite.

Similar results have been obtained in a number of animals. A representative experiment is plotted in Figure 4. One hind foot was frostbitten for 3 minutes at − 55° C. Application of cold to the ears at "A" and to the body at "D" resulted in a temperature drop in the toes of the normal foot of 8.5° and 12° C. respectively, while the toes of the frostbitten foot showed much less change in temperature. Application of heat to the ears at "B" and general body warming at "C" and "E" resulted in marked rise in temperature of the normal toes. It is apparent (Figure 4) that after the first 1½ hours there is a tendency for changes in rectal temperature to be accompanied by changes in skin temperature of the toes of the frostbitten foot.

The temperature changes in the toes of the animal illustrated in Figure 4 and in other animals studied are brought about by changes in blood flow through the tissues. Tittel (5), by direct observation of rabbit ears frozen with solid carbon dioxide, has described a state of passive dilatation of arterioles in the injured region of the ear. This passive dilatation should be regarded as a relative dilatation, and, although constriction may not occur, the size of the vessels may be altered by changes in perfusion pressure. If the arterioles within the injured regions of the feet are in a state of passive dilatation similar to that described by Tittel in rabbit ears following freezing, then blood flow and hence skin temperature should be determined by the perfusion pressure and the patency of the arteries proximal to the level of injury. The rise of skin temperature in uninjured regions following general application of heat demonstrates the existence of adequate systemic arterial pressure. In the injured limb both the failure of signs of vasoconstriction after chilling and of vasodilatation after warming could be explained on the basis of severe spasm of the arteries proximal to the level of injury. Such arterial spasm has been described by Burdenko (6) in human frostbite of the feet where arterial spasm reached as high as the common iliacs. The measurements given in Figure 4 show that the temperatures of the toes of the frostbitten foot were persistently lower than the toes of the normal foot. The course of cooling of the toes in the injured foot shows that there was a progressive reduction of blood flow from 45 minutes after injury to about 2½ hours after injury. The slope of this line differs little from that describing the fall in temperature of the normal toes when vasoconstriction was induced by the application of cold. It is therefore probable that at the time of measurement, spasm of large arteries on the injured side was occurring and that the spasm was not capable of being relaxed by the usual reflex responses to the application of heat elsewhere on the body.

It should be emphasized that the abnormalities of thermovascular response described above are not interpreted as indications of later vasomotor abnormalities, but are restricted to the first few hours after injury. Studies of skin temperature over a longer period after injury (Figure 3) show that the temperature of the toes of rabbit feet injured by cold tend to stabilize at about 28° C. and that there is usually a slight rise until 48 to 50 hours after injury. The application of cold as illustrated in Figure 4 brought about sufficient reduction of blood flow to bring the toe temperature of the injured foot to approximately 24° C. while that of the uninjured foot did not fall below 29.5° C. Thus it is suggested that vasoconstrictor responses to cold may be potentiated locally by recent cold injury but that central inhibition of vasoconstrictor discharges over the vasomotor neurons supplying the vessels of the injured extremity is not evoked by the local or general application of heat.

SUMMARY

Rabbits' feet and ears, frostbitten by immersion in liquid at temperatures from − 15° to − 70° C. for varying lengths of time, undergo a series of changes which, for a given time and temperature, are constant and reproducible. Upon removal from the cold liquid the part is solidly frozen, but is not brittle. Massive edema develops with the

reestablishment of blood flow to the part. After 3 to 4 days, in those instances in which the injury is of sufficient severity, wet gangrene develops. Drying begins 4 to 6 days after injury, resulting in mummification of the foot or ear which eventually separates near the line to which it was immersed in the freezing mixture.

Observations of the skin color of the injured region and measurements of skin temperature indicate that for a time after injury blood flows through the injured region, often at a faster rate than in the normal foot or ear. During the first 50 hours after injury there is a progressive fall in skin temperature of frostbitten ears, until a level approximately equal to room temperature is reached coincidentally with the occurrence of wet gangrene. Comparison of skin temperature of the toes of frostbitten feet with the temperature of the normal foot indicates that in those animals in which the injury is of sufficient severity to result in subsequent loss of the toes, there is a significant decrease in temperature of the injured foot below that of the normal foot during the first 6 hours after injury. This difference becomes more marked with time.

BIBLIOGRAPHY

1. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. I. A method for producing gangrene by means of controlled injury by cold. J. Clin. Invest., 1947, **26**, 229.
2. Ariev, T. Y., Experimental observations on the brittleness of frozen extremities. Vestn. Khirur., 1937, **52**, 63.
63.
3. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. III. Edema following cold injury: its magnitude and the composition and source of edema fluid. J. Clin. Invest., 1947, **26**, 245.
4. Fisher, R. A., Statistical Methods for Research Workers. Ed. 6, London, Oliver and Boyd, 1936, p. 130.
5. Tittel, S., Ueber die Reaktionsweise des Gefässsystems bei lokaler Erfrierung. I. Die Art des Reaktionsablaufes an den einzelnen Abschnitten des Gefässsystems. Ztschr. ges. exper. Med., 1943, **113**, 698.
6. Burdenko, N. N., The effect of frostbite on the sympathetic nervous system. Amer. Rev. Sov. Med., 1943, **1**, 15. From Khirurgia, 1942, No. 5–6, 3.

STUDIES ON GANGRENE FOLLOWING COLD INJURY. III. EDEMA FOLLOWING COLD INJURY: ITS MAGNITUDE AND THE COMPOSITION AND SOURCES OF EDEMA FLUID[1]

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One of the most prominent features of severe cold injury (frostbite) is the early and rapid development of edema of the injured part. We have described previously a method for the production of controlled injury by cold (1) and the general course of events ending in gangrene (2). The data presented here concern the edema following frostbite. Most of these observations were carried out during the first 24 hours after injury, at a time when swelling of the injured part occurs. The method used for the production of the injury by cold is the same as that described previously (1).

A. Magnitude and location of edema

The maximum increase in volume of ears and feet of rabbits following frostbite of different duration and at different temperatures is given in Table I. In ears the greater part of the fluid is found in the more distensible proximal part. The measurements were made by immersing as much as possible of the ear in water and measuring the volume of water displaced. In the leg most of the measurements were made as far proximally as the part was frozen (to the tuberosity at the base of the fifth metatarsal). The fluid, however, is not confined to this region, but is forced up the leg in a manner similar to that observed in ears. A few measurements of volume were made as far as the tuber calcanei (Table I). Twenty-four hours after injury of the foot, edema is apparent above the ankle and in some animals extends as far as the knee. Measurements of total fluid loss by estimation of the volume change of the entire leg were not made.

The appearance of the foot of a rabbit 2 hours after frostbite (3 minutes at $-55°$ C.) is shown

TABLE I

Swelling of feet and ears following frostbite

Duration of immersion	Temp.	Initial volume	Maximum volume	Increase	$\frac{\text{Max. vol.}}{\text{Init. vol.}} \times 100$
min.	*° C.*	*ml.*	*ml*	*ml.*	
Rabbit ears					
1½	−55	7.5	36	28.5	480
		9.0	39	30	433
Rabbit feet	(Volume measured to base of fifth metatarsal)				
1	−55	12	25	13	208
		13	33	20	254
		13	25	12	192
		13	31	18	238
		14	20	6	142
		13	28	15	215
		12	25	13	208
2	−55	13	36	23	277
		13	37	24	285
		13	25	12	192
		12	30	18	250
		11	31	20	282
3	−55	12	35	23	291
		11	30	19	273
		12	30	18	250
		13	35	22	269
		14	35	21	250
3	−15	11	12	1	109*
		9	9	0	100*
3	−25	11	27	16	245
		10	30	20	300
3	−35	13	31	18	239
		12	33	21	275
3	−45	12	28	16	233
		11	30	19	273
60	−15	12	34	22	283
		13	32	19	246
		13	30	18	250
Rabbit feet	(Volume measured to tuber calcanei)				
3	−55	20	65	45	325
		17	40	23	235
		27	75	48	277

* Freezing did not occur.

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

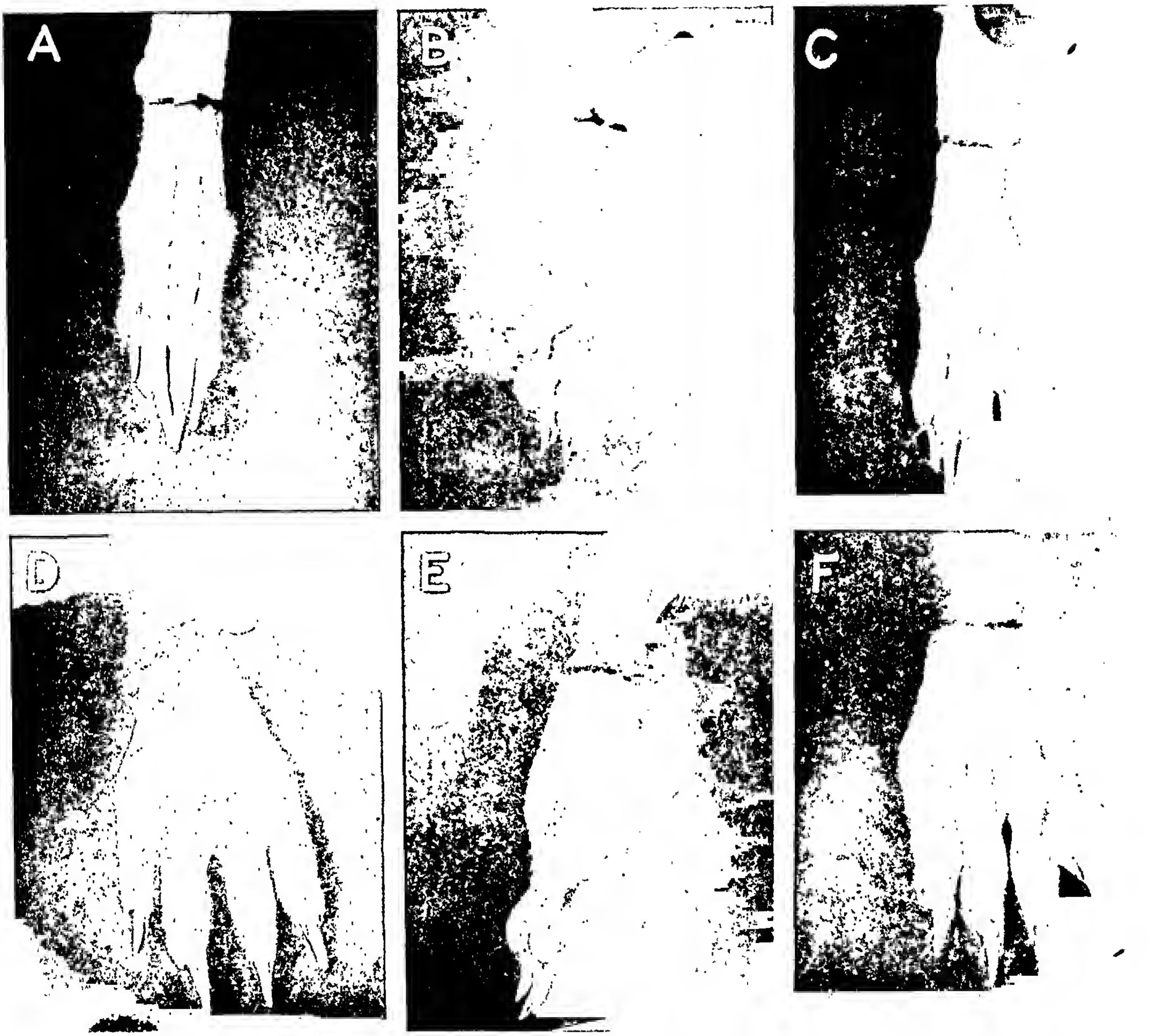

FIG. 1. EDEMA OF RABBIT FEET FOLLOWING SEVERE COLD INJURY

A, B and C show a right hind foot, with hair removed, before cold injury. D, E and F show the same foot 2 hours after injury by immersion for 3 minutes at − 50° C. Foot immersed to the point indicated by the ink line. Volume of edematous foot is 2.7 times that of the foot before injury.

in Figure 1. The toes are spread apart by the accumulation of fluid, and swelling is especially marked on the dorsal surface of the foot. Much less swelling is evident on the plantar surface where the skin is much more tightly bound to the underlying connective tissue. Upon incision of the dorsum of an edematous foot, the edema fluid may be seen to be distributed chiefly in the loose connective tissue underlying the skin. The fluid itself is viscous and drains slowly from the incision. It coagulates within a few minutes after withdrawal from the foot.

In order to determine whether any exudation of fluid occurred in the proximal, uninjured portion of a frostbitten ear, the distal, frostbitten part was amputated after injury in one experiment. Following frostbite for 90 seconds at − 55° C. a clamp was placed on the ear just proximal to the line of immersion. Two and one-half hours later the ear distal to the clamp was amputated, cauterized, and the clamp then released. On the following day there was very slight edema present in the distal part of the stump near the site of amputation. This had disappeared on the second day. No

edema approaching in magnitude that occurring after untreated frostbite was observed, and no evidence of edema was seen in the proximal part of the ear. It thus appears that the fluid loss occurs only in the distal injured part of the ear and that edema proximal to the frostbitten region is the result of forcing of fluid into that region after the limit of distensibility of the tip of the ear is reached.

B. Rate of swelling

In all animals in which the foot or ear actually became frozen during immersion (true frostbite), thawing and return of blood to the injured extremity were followed immediately by rapid and extensive swelling. Increase in the volume of a frostbitten foot could usually be demonstrated 20 minutes after injury. The relative increase in volume of feet frostbitten for 1, 2, and 3 minutes at − 55° C. may be compared in Figure 2, in which the maxima and minima for each duration of injury are plotted at various time intervals after frostbite. The volume was measured to the line of immersion (the tuberosity at the base of the fifth metatarsal).

If comparisons are made between rabbit feet immersed at − 55° C. for 1, 2, and 3 minutes, it is found that the rate of swelling increases with the duration of immersion and thus also with the severity of the injury. Although swelling begins within 20 to 30 minutes after exposure in all cases, it occurs most rapidly following 3-minute exposure and least rapidly after a 1-minute exposure. Following 1-minute immersion the maximum volume is reached in from 3 to 6 hours after injury. Following a 2-minute immersion the maximum occurs somewhat earlier, and after 3-minute immersion the maximum volume is attained in 1 to 2 hours in some animals. The maximum volume attained is similar following the different periods of immersion, although some feet showed only slight increase in volume following 1-minute exposure and others became as swollen as those exposed for 3 minutes. The great variability of the foot volume following 1-minute exposure (Figure 2) is an indication of lack of

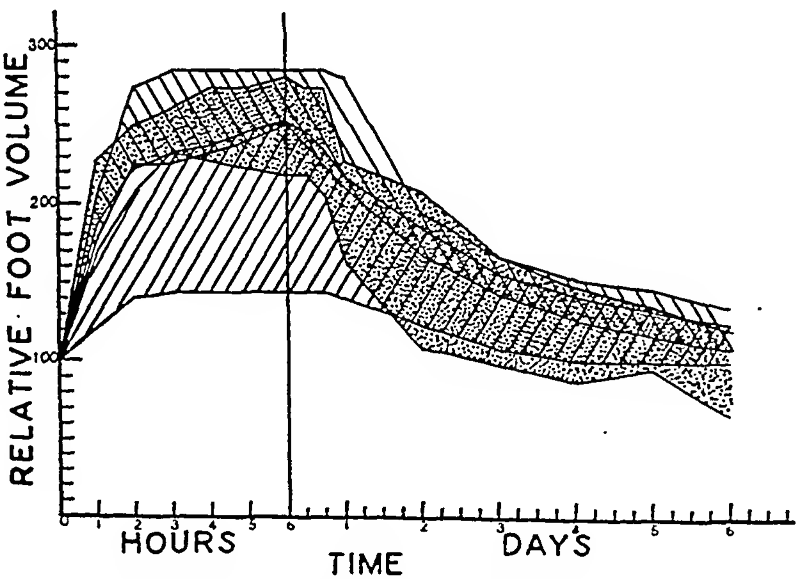

FIG. 2. COMPARISON OF VOLUME CHANGES IN RABBIT FEET AFTER EXPOSURE AT − 55° C. FOR ONE, TWO, AND THREE MINUTES

Initial foot volume expressed as 100. For each severity of injury the maximum and minimum values of foot volume at different time periods after injury are connected by straight lines. The area between the curves of maximum and minimum volume include all of the cases measured. Area shaded right—1-minute immersion. Area shaded left—2-minute immersion. Area spotted—3-minute immersion.

uniformity in injury following this length of immersion.

The decrease in foot volume begins about 1 day after injury and appears to be more rapid following the more severe injury. This decrease in volume results from dehydration of the foot, due to loss of fluid to the outside as well as internally. The external water loss is most rapid when necrosis and wet gangrene appear, and, since necrosis of most of the injured area is an invariable consequence of a 3-minute immersion at $-55°$ C., drying and shrinkage of the foot below its normal size are most rapid in this type of injury.

C. *Subcutaneous tissue pressure*

Subcutaneous tissue pressures in frostbitten feet were determined at varying times after injury by the method of Henderson (3) as modified by Wells, Youmans, and Miller (4). The needle was inserted through the skin on the dorsal surface of the foot into the edematous mass of underlying tissue, and the point adjusted so that it was approximately midway between the skin and metatarsal bones. Care was taken that only two punctures were made in each foot; repeated puncturing of the skin resulted in loss of fluid through the skin and consequent decrease in pressure.

The results of determinations of subcutaneous tissue pressure in the frostbitten feet of 9 animals

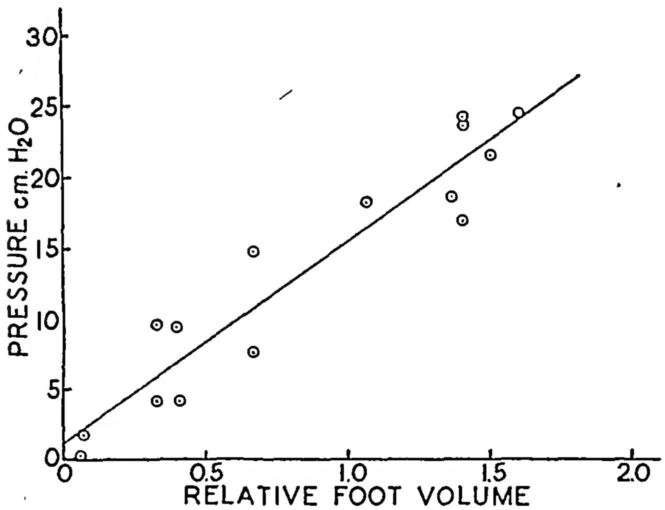

FIG. 3. RELATIONSHIP BETWEEN FOOT VOLUME AND SUBCUTANEOUS TISSUE PRESSURE AFTER FROSTBITE

Subcutaneous tissue pressure in cm. H_2O plotted as a function of relative increase in foot volume after exposure of one foot at $-55°$ C. for 3 minutes. Data from 9 animals. A maximum of two determinations was made on each animal.

are plotted against the foot volume at the time of pressure measurement in Figure 3. In all cases the frostbite was produced by immersion at $-55°$ C. for 3 minutes. The line drawn in Figure 3 was fitted to the data by the method of least squares and may be represented by the equation

$$P = 1.12 + 0.144\ V \qquad (1)$$

in which P is subcutaneous pressure in cm. of water and V is the relative increase in foot volume.

It may be seen from Figure 3 that as the volume of the foot increases the pressure is found to increase until, at maximum foot volume, the subcutaneous pressure is approximately 25 cm. H_2O. This pressure is of the same order of magnitude as the pressure inside capillaries. Landis (5, 6) found by direct measurement that the capillary pressures of arteriolar and venous capillaries were 30 cm. H_2O and 17 cm. H_2O respectively in the mesentery of the rat and 38.5 cm. H_2O and 17 cm. H_2O in the mesentery of the guinea pig. The importance of high subcutaneous pressure in relation to the functional pathology of frostbite will be discussed later.

When the foot has attained a volume 250 to 300 per cent of the initial volume, further increase measured to the line of immersion does not occur, but the pressure within the foot reaches such a level that fluid is forced in a proximal direction above the level of injury.

If the time course of increase in subcutaneous pressure in the foot following cold injury is considered, it is found that the pressure increases during the period of swelling (for the first 4 to 6 hours), and then reaches a maximum pressure coinciding in time with the maximum foot volume.

D. *Composition of edema fluid*

Determination of the protein concentration of edema fluid withdrawn from frostbitten feet and in fluid drawn from blisters on frostbitten ears was made at various times after frostbite. The protein concentration was calculated from the specific gravity of the fluid determined by the falling drop method (7). Protein concentration was calculated from the formula given by Weech (8):

$$Pr = (\text{Sp. Gr.} - 1.0069) \times 340.1 \qquad (2)$$

in which Pr is the protein concentration in grams per 100 ml.

1. *Protein concentration of ear blister fluid*

The protein concentration of blister fluid from frostbitten ears, withdrawn at various times after frostbite of 90-second duration at − 55° C., is given in Figure 4. In the greater number of these animals the blisters formed in the first 4 hours after injury, although in some cases blisters were not seen until 8 to 12 hours after injury. Fluid from the blisters was straw-colored and did not contain appreciable amounts of hemoglobin. It may be seen from the figure that there is a decrease in protein concentration of blister fluid with time. The line drawn in the figure, fitted to the data by the method of least squares, may be represented by the equation

$$Pr = 4.303 - 0.0254\ t \tag{3}$$

in which Pr is the protein concentration in grams per 100 ml. and t is the time in hours after frostbite. The coefficient of correlation between protein concentration and time after frostbite was calculated to be − 0.798.

Pochin (9) found a negative correlation between protein concentration and time after injury in edema fluid taken from rabbit ears following vascular occlusion. The initial protein concentration of the edema fluid after vascular occlusion and of blister fluid after frostbite injury are very

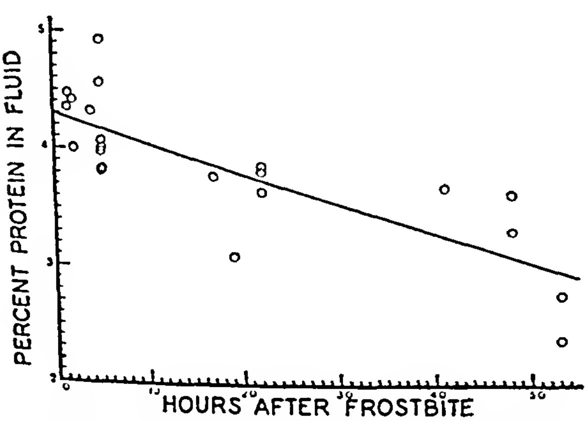

FIG. 4. TIME COURSE OF CHANGE IN PROTEIN CONCENTRATION OF EAR BLISTER FLUID FOLLOWING SEVERE COLD INJURY

Data from 12 animals. Frostbite was produced in all cases by immersion at − 55° C. for 90 seconds.

TABLE II

Protein concentration in edema fluid from frostbitten feet
Exposure at − 55° C.

No. of animals	Duration of exposure	Time after frostbite at which fluid was sampled	Mean protein	S. E. of mean
			per cent	
5	1	120–240 minutes	3.46	.668
22	3	90–240 minutes	4.13	.139
4	3	24 hours	3.01	.323
4	3	44 hours	3.91	.585

similar (4.5 per cent and 4.3 per cent respectively). Pochin's data show a more rapid decrease in protein concentration in the edema fluid after occlusion than that reported here in blister fluid after frostbite. This may indicate a more rapid removal of protein-containing fluid via the lymphatics in occlusion.

2. *Protein concentration of foot edema fluid*

The protein concentration of edema fluid taken from frostbitten feet under various circumstances is given in Table II. It may be seen from the table that the protein concentration after 1-minute frostbite is somewhat less than after 3-minute frostbite at − 55° C. This difference is, however, not significant ($P < 0.7$).

The data for the protein concentration of foot edema fluid taken at different times after injury are not sufficient to establish so definite a relationship as that in ear blister fluid. The correlation between protein concentration and time in hours after injury was − 0.494 and the straight line fitted to the data may be represented by the equation

$$Pr = 4.326 - 0.052\ t \tag{4}$$

in which the symbols are the same as those used in equation (3). This relationship is based upon 22 determinations made between 1 and 6 hours after injury and upon 4 determinations made 24 hours after frostbite. It is apparent that in frostbitten feet, as in frostbitten ears, the protein concentration of the edema-fluid is initially high, only slightly below that of the plasma, and that it decreases progressively with time after injury.

Table II also shows the protein concentration of foot edema fluid taken 44 hours after frostbite. In this case there was an apparent increase in protein concentration. It seems probable that this

increase is due to loss of water more rapid than loss of protein from the foot.

The protein concentration found in the foot edema fluid and in ear blister fluid following cold injury is in good agreement with the data reported by Harkins and Harmon (10) for protein concentration in edema fluid from severely frostbitten dogs. They report a mean edema fluid protein concentration of 3.6 per cent.

3. *Effect of edema fluid upon blood pressure and isolated smooth muscle.*

The frequent reports of the presence of a toxic substance in plasma and edema fluid after burns (11, 12) suggest that such toxic materials may be present in the edema fluid taken from extremities following cold injury. In order to test this possibility, edema fluid removed from frostbitten rabbit feet 2 hours after injury was examined in the following manner.

a. Effect upon blood pressure. Edema fluid was withdrawn from the feet of two rabbits 85 and 95 minutes after frostbite. In one case the fluid was used immediately and in the other it was held overnight in a refrigerator. This fluid was then injected intravenously into rabbits anesthetized with dial. Arterial pressure was recorded during and following the injection. Injection of the edema fluid in amounts up to 2 ml. in a rabbit weighing 2.4 kgm. produced no change in the level of arterial pressure.

b. Effect upon isolated guinea pig intestine. Segments of guinea pig ileum were suspended in oxygenated Tyrode solution containing 1:1,000,000 atropine. Addition of edema fluid withdrawn from frostbitten rabbit feet produced slight contraction in one instance. In this case 1.0 ml. of edema fluid was equal in activity to 0.1 μg. histamine. This activity may be accounted for entirely on the basis of the normal histamine content of the small amounts of blood present in the edema fluid, since rabbit blood contains 10 to 12 μg. histamine per ml. (13). In all muscle preparations the activity of the muscle was confirmed by contraction in response to additions of histamine.

c. Assay of edema fluid for histamine. Edema fluid withdrawn from frostbitten feet 2 to 4 hours after injury was prepared for histamine assay by the method of Barsoum and Gaddum (13) as modified by Anrep *et al.* (14). The extracts were tested upon the atropinized guinea pig ileum and upon atropinized rectal caecum of the fowl. No contractions of the isolated muscle were produced by the edema fluid extracts. Comparison with known concentrations of histamine showed that the histamine content of the edema fluid must be less than 0.2 μg. per ml. of original edema fluid.

d. Intradermal injection of edema fluid. Edema fluid from a rabbit foot, withdrawn 95 minutes after injury, was injected intradermally into the ears of two rabbits. The volumes injected were approximately 0.1 and 0.3 ml. Slight vasodilatation was seen immediately surrounding the sites of injection within 5 minutes. No edema developed and the slight hyperemia disappeared within 12 hours.

Histamine liberation from perfused limbs injured by high temperature has been described by Kellaway and Rawlinson (15). The edema fluid formed after frostbite, however, does not contain significant amounts of histamine. Failure to demonstrate the presence of toxic materials in this edema fluid by the methods outlined above is not interpreted to mean that chemical substances are not implicated in the local changes following frostbite. It is possible that such principles as those described by Shorr, Zweifach and Furchgott (16), which would not be detected by the methods used here, may play a part in the local circulatory changes following cold injury.

E. *Changes in blood composition coincidental with swelling*

1. *Plasma protein changes following cold injury.*

Plasma protein concentration was calculated from the plasma specific gravity, determined by the falling drop method of Barbour and Hamilton (7), by means of equation (2). In every case the plasma protein concentration decreased during the period of swelling of the frostbitten foot. The results obtained in a representative experiment are shown in Figure 5 (which also shows changes in foot volume, hematocrit, hemoglobin concentration, and blood specific gravity during the same time period). In this experiment the plasma protein concentration fell from an initial value of 5.34 per cent to a minimum of 3.96 per cent. Similar results were obtained in 13 other animals in which the plasma protein fell to 81.3 per cent of the initial 185 to 357 minutes after injury. Plasma protein concentrations before frostbite and at times ranging from 160 to 357 minutes after frostbite in 6 animals are given in Table III. It may be seen that in all cases there is a decrease in plasma protein concentration following frostbite, ranging from 0.98 to 1.57 grams per 100 ml. The relationship between the decrease in plasma protein concentration and the protein concentration in edema fluid will be considered below.

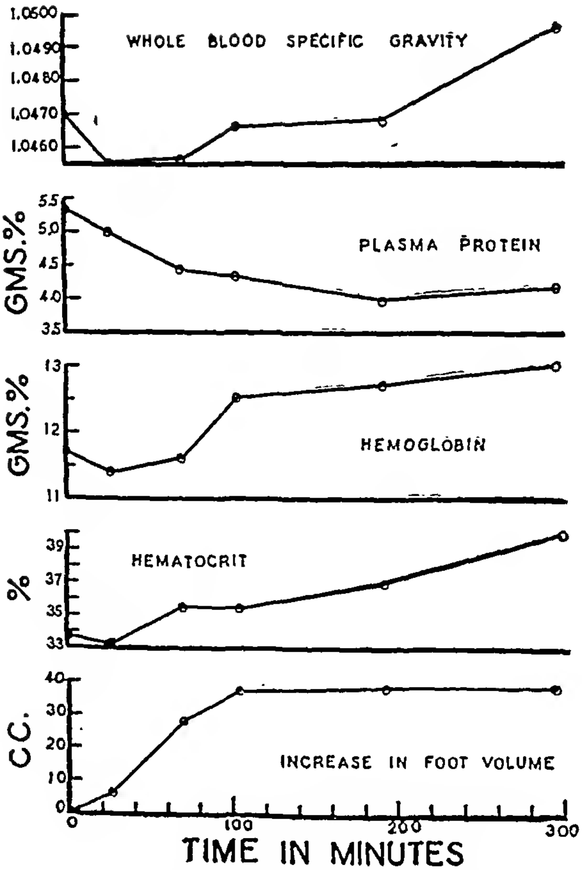

FIG. 5. TIME COURSE OF CHANGES IN WHOLE BLOOD SPECIFIC GRAVITY, PLASMA PROTEIN, HEMOGLOBIN, HEMATOCRIT AND FOOT VOLUME FOLLOWING SEVERE COLD INJURY

Representative data from one animal after exposure of one hind foot at − 55° C. for 3 minutes.

In order to determine the approximate duration of the decrease in plasma protein concentration following this type of frostbite, determinations were made 24 hours after injury in four animals. The results are given in Table IV. It is clear from the data given in the table that after this length of time the plasma protein concentration had returned to approximately the initial value. The data show no tendency toward a decrease in plasma protein concentration such as might result from leakage of protein into regions remote from the site of injury.

TABLE III

Plasma protein changes following cold injury
3-minute immersion of foot at −55° C.

Animal weight	Initial plasma protein	Time after injury of second determ.	Increase in foot volume	Second plasma protein
kgm.	*grams per 100 ml.*	*min.*	*ml.*	*grams per 100 ml.*
2.59	4.90	185	35*	3.33
1.76	5.03	225	20	4.05
2.40	5.24	160	30*	4.00
2.50	5.35	192	38*	3.98
		295	38*	4.15
2.50	6.25	322	25	5.24
2.80	6.43	357	35*	5.34

* Volume increase measured to the tuber calcanei.

TABLE IV

Plasma protein concentration one day after frostbite
Feet exposed at −50° C. to −54° C. for 3 minutes

Initial plasma protein	Time after frostbite	Plasma protein	Difference
grams per 100 ml.	*hours*	*grams per 100 ml.*	
4.83	23	4.69	−0.14
4.60	24	4.15	−0.45
4.00	24	3.81	−0.19
4.63	24	4.76	+0.13

2. *Relationship of plasma protein changes to edema fluid protein concentration*

Information regarding the origin of the edema fluid in frostbitten extremities may be obtained by comparison of the protein content of the edema fluid with the plasma protein concentration before and after injury. Such a comparison has been made for the data given here on two bases: (1) comparison of the mean protein concentration of the edema fluid with the mean initial plasma protein concentration; (2) comparison, in individual animals, of the loss in total circulating protein with the total protein found in edema fluid.

Determination of the initial plasma protein concentration (before frostbite) in 23 animals gave a mean value of 5.42 grams per 100 ml. (Range 4.00 to 6.56; S.E. of mean = 0.137). The protein concentration of edema fluid taken from frostbitten feet and from blisters on frostbitten ears during the first few hours after injury were given previously as 4.13 grams per 100 ml. and 4.23 grams per 100 ml. respectively. If these values for protein concentration in the edema fluid are compared directly with the mean plasma protein concentration it may be calculated that for feet 76.2 per cent or for ears 78.0 per cent of the edema fluid is whole plasma and

TABLE V

Protein content of edema fluid

Calculated data derived from experimental data in preceding table

1	2	3	4	5	6	7
Animal weight	Calc.* plasma volume	Foot volume increase	Decrease in plasma protein (determined)	Grams protein lost	Protein conc. of edema fluid	
					Theoretical	Observed
kgm.	*ml.*	*ml.*	*grams per 100 ml.*		*grams per 100 ml.*	
2.59	103.6	35	1.57	1.63	4.66	3.6
1.76	70.4	20	.98	.69	3.45	
2.40	96.0	30	1.24	1.19	3.97	3.8
2.50	100.0	38	1.37	1.37	3.61	
	100.0	38	1.20	1.20	3.16	3.1
2.50	100.0	25	1.01	1.01	4.04	4.3
2.80	112.0	35	1.09	1.22	3.49	5.3

* Four per cent of body weight.

the remainder must therefore be plasma ultrafiltrate.

The data given in Table III make it possible to calculate, for individual animals, the loss of total circulating protein as compared with protein in edema fluid from the foot of the same animal. In Table III are given the initial plasma protein concentration, and, after 160 to 357 minutes, the increase in foot volume and the plasma protein concentration at that time. In Table V are listed certain derived data from Table III, as well as the actual protein concentration of the foot edema fluid from these animals. The normal plasma volume of these animals is assumed to be four per cent of the body weight. It may be seen that the loss in total circulating protein ranges from 0.69 to 1.63 grams if it be assumed that no change in plasma volume occurs (Column 5). When this is divided by the volume of fluid measured to have accumulated in the foot during the time period given (Column 3), the theoretical protein concentration of the edema fluid may be calculated (Column 6). Good agreement is seen between this theoretical value and that actually determined (Column 7), indicating that the total loss of circulating protein may be accounted for by protein occurring in the edema fluid. It should be pointed out that this comparison involves, at best, a rough approximation of the actual changes. The determination of the exact volume of edema fluid cannot be made with great accuracy by the displacement method. Furthermore the assumption that no change in plasma volume occurs is not strictly valid, since data presented below indicate a reduction in plasma volume soon after injury, with a progressive replacement in plasma volume over a period of several hours.

3. *Changes in hemoglobin concentration and hematocrit during swelling.*

The hemoglobin concentration and the hematocrit were determined in arterial blood during the period of swelling of the foot following a 3-minute immersion at − 55° C.

The procedure was as follows: Heparin was used as an anticoagulant. Hemoglobin was determined by the acid hematin method using 0.05 ml. of blood. Hematocrit was determined in Van Allen tubes without dilution of the blood. In several animals the hematocrit was calculated from the hemoglobin concentration using a value of 33.9 grams hemoglobin per 100 ml. of packed erythrocytes (17). This was calculated assuming an oxygen capacity of 46.1 ml. of oxygen per 100 ml. packed cells (17) and 1.36 ml. oxygen to be bound by 1 gram hemoglobin (18). In a limited number of determinations on rabbit blood the mean corpuscular hemoglobin concentration was found to be 33.6 grams per 100 ml. of packed erythrocytes.

For the four animals in which both hemoglobin and hematocrit were determined on the same samples of blood, the mean corpuscular hemoglobin concentration was calculated for the initial sample and the sample taken at the time of attainment of maximum foot volume. It was found that a decrease occurred in two animals (34.7 to 32.8 and 35.3 to 33.5) and no change occurred in the other two. Since the difference found was not great and appeared in only two of four animals, swelling of erythrocytes during the development of edema is not considered to be an important or invariable consequence of cold injury under the conditions used here.

The hemoglobin and hematocrit values, together with the figures showing fluid loss to the injured foot and the whole blood specific gravity, are given in Table VI. As is to be expected from data given previously which indicated loss of about 77 per cent whole plasma, the hemo-

TABLE VI

Changes in hemoglobin concentration and hematocrit following frostbite of rabbits' feet

Feet exposed at −55° C. for 3 minutes

Animal number	Time after frostbite	Increase in foot volume	Whole blood spec. gr.	Hemo-globin	Hemato-crit
	min.	*ml.*		*grams per 100 ml.*	*per cent*
45	0	0	1.0470	11.7	33.7
	26	6	1.0455	11.4	33.2
	70	28	1.0456	11.6	35.5
	104	37	1.0466	12.5	35.4
	192	38	1.0468	12.7	36.9
	295	38	1.0495	13.0	39.7
3	0	0	1.0560	14.1	41.5*
	76	18	1.0570	14.9	
	183	22	1.0580	16.4	
	316	23	1.0576	16.1	47.5*
13	0	0	1.0550	14.3	42.5*
	37	6	1.0554	14.8	
	68	10	1.0564	15.8	
	174	15	1.0577	16.1	
	223	18	1.0569	15.4	45.6*
7	0	0	1.0599	16.8	49.5*
	203	15	1.0619	17.3	
	246	16	1.0622	17.4	51.5*
19	0	0	1.0504	11.6	35.0
	216	21	1.0510	13.5	41.0
17-s	0	0	1.0495	11.8	36.0
	190	30	1 0495	12.9	38.5
6-s	0	0	1.0533	13.6	38.5
	200	36	1.0521	14.3	42.7

Number 45, 17, 6: Foot volume measured to tuber calcanei.

* Hematocrit calculated from hemoglobin (see text).

s = spleen removed from circulation before frostbite.

globin concentration and hematocrit are found to increase progressively during the period of swelling. These values are plotted in Figure 5 for one representative animal.

From a comparison of the initial and final hematocrit values, provided the loss of fluid to the injured foot is known, it is possible to calculate the extent to which plasma volume, reduced due to loss into the injured region, has been replaced by withdrawal of fluid from extravascular sources. These data are shown in Table VII. The cell volume is assumed to remain constant throughout the experiment. In two animals (Nos. 17 and 6) the spleen was excluded from the circulation before frostbite in order to exclude the addition of cells from this source. These animals did not appear to differ from the others in the series.

TABLE VII

Calculations showing estimated replacement of plasma volume following severe cold injury

See text for calculations and meanings of symbols used

Animal number	45	3	13	7	19	17*	6*
Body weight (kgm.)	2.50	2.66	2.88	2.88	2.20	2.50	3.20
Initial V_b (ml.)	175	186	199	199	154	175	224
Initial Ht	33.7	41.5	42.5	49.5	38.0	36.0	38.5
V_c (ml.)	89	77	88	97	54	63	86
Initial V_p (ml.)	116	109	114	102	100	112	138
Time after injury (min.)	295	316	223	246	216	190	200
Fluid loss (X) (ml.)	38	33	24	23	30	30	36
Calc. final V_p (ml.)	78	76	90	79	70	82	102
Calc. final V_b (ml.)	137	153	175	176	124	145	188
Calc. final Ht	43.1	80.4	48.3	58.4	43.6	43.5	45.8
Final Ht (determined)	39.7	47.5	45.6	51.5	41.0	38.5	42.7
Final V_b (ml.)	149	162	185	189	132	164	202
Replacement of plasma vol. (ml.) (Final V_b − calc. final V_b)	12	9	10	13	8	19	14

* Spleen occluded from the circulation before cold injury.

For Table VII the calculations involved are as follows: initial blood volume (Initial V_b) is assumed to be 7 per cent of the body weight, from which is calculated the initial cell volume (V_c) and the initial plasma volume (Initial V_p) by use of the hematocrit determined initially (Initial Ht).

$$V_c = (\text{Body weight} \times 0.07) \times \text{Initial } Ht$$
$$\text{Initial } V_p = (\text{Body weight} \times 0.07) \times (100 - \text{Initial } Ht)$$

If it be assumed that the fluid lost (X) into the injured region is plasma and that the plasma volume is reduced by the amount (X), then the following calculated values may be derived:

$$\text{Calc. Final } V_p = \text{Initial } V_p - X$$
$$\text{Calc. Final } V_b = \text{Cal. Final } V_p + V_c$$
$$\text{Calc. Final } Ht = \frac{V_c \times 100}{\text{Calc. Final } V_b}$$

If, now, the final determined hematocrit value (Final Ht) be compared in each case with the final calculated hematocrit (Calc. Final Ht), it is found that the determined hematocrit value is smaller in all the animals. This indicates that part of the plasma lost has been replaced by water withdrawn from the interstitial compartment and from the gut. The extent of this replacement may be estimated by calculation of the blood volume on the basis of the determined final hematocrit (Final Ht) and comparison of this figure with the calculated final blood volume (Calc. Final V_b) derived above.

$$\text{Final } V_b = \frac{\text{Calc. Final } Ht \times \text{Calc. Final } V_b}{\text{Final } Ht}$$

It is found (Table VII) that the replacement of plasma volume amounts to from 8 to 19 ml. in the time interval studied.

An examination of the hematocrit values following cold injury thus indicates a reduction in plasma volume which is partially replaced by extravascular fluid within a few hours. Further evidence for this is given in the next section.

4. *Measurement of plasma volume following cold injury*

In four animals the plasma volume was determined by the dye method immediately before frostbite and again after 2 to 4 hours. Preliminary experiments showed that intravenously injected T–1824 was readily lost from the circulation into the injured foot, so that the frostbitten foot must be excluded from the circulation before the second plasma volume determination is made in order to obviate fictitiously high values.

The procedure was as follows: Initial plasma volume was determined by the intravenous administration of 2 mgm. per kgm. T–1824; the determination of the dye concentration in plasma was made after 10 minutes (19). Plasma volume after frostbite was determined after first occluding the circulation to the injured foot by a tourniquet placed just above the knee. A blood sample was then withdrawn to serve as a control, an additional injection of 1 mgm. per kgm. T–1824 was made intravenously, and the dye concentration again was determined after 10 minutes. Concentrations of T–1824 were determined in undiluted plasma using a 620 μm. filter in a Klett-Summerson photoelectric colorimeter.

Table VIII shows the results of determinations of initial plasma volume and plasma volume after frostbite of one hind foot at − 55° C. for 3 minutes. It will be seen that the determinations made 120, 148, and 180 minutes after injury all show reductions in plasma volume, but that after 240 minutes (in one animal) the plasma volume is made up to its initial value. These results confirm those obtained by calculation of hematocrit differences in showing that the fluid lost from the plasma is rapidly replaced during the first few hours after injury.

TABLE VIII

Plasma volume before and after severe cold injury

Plasma volume was determined by the use of T-1824 as described in the text

Animal number	82-B	12	82	79
Weight (kgm.)	3.855	4.044	4.095	1.760
Initial hematocrit	38	43	43	39
Final hematocrit	44	49	46	38
Time after injury of second plasma volume measurement (min.)	120	148	180	240
Increase in foot volume (ml.)	28	27	38	20
Initial plasma volume (ml.)	172	183	156	97
Final plasma volume (ml.)	143	117	125	98
Difference (ml.)	−29	−66	−31	+1

5. *Oxygen and hemoglobin concentration in blood flowing from frostbitten limbs.*

These determinations were made on samples of blood withdrawn from the anterior tibial vein following immersion of the foot for 3 minutes

TABLE IX

Venous and arterial blood hemoglobin concentration and oxygen content following severe cold injury

Animal number	Time after frost-bite	Foot volume increase	Oxygen content		Hemoglobin	
			Venous	Arterial	Venous	Arterial
	min.	*ml.*	*vol. per cent*	*vol. per cent*	*grams per cent*	*grams per cent*
5	0		14.9	17.9		
	15		16.4			
	60		16.9			
11	0	0	11.4	14.8		
	97		18.1			
	115			17.0		
	135		16.6			
	150			16.1		
	210		13.8			
	232		13.0			
	265			16.3		
	294	37				
4	0	0	15.5	17.6		
	20			21.9		
	33	6	21.4			
	44			18.9		
	59	15				
	68		23.9			
	78			18.1		
	147		15.5			
	158	23		22.0		
3	0	0	14.8	16.2	14.1	14.1
	26		16.2			
	46			18.1		14.0
	58				15.2	
	62	14	17.2		15.2	
	76	18		22.4		14.9
	96	21	19.2		17.2	
	183			21.8		16.4
	211		19.1	20.0	17.0	
	268		19.3		16.9	
	316	23		19.7		16.1
13	0	0	13.7	16.6	14.8	14.3
	20	2	15.4		14.9	
	37			17.2		14.8
	56			18.0		15.8
	68		16.1			
	80	11			15.9	
	163			18.0		
	174			19.2	16.1	
	198		22.2			
	223	18		16.9	15.8	
7	0	0	18.2	19.0	17.4	16.8
	133	4				
	186		18.2		18.9	
	203	14		21.9		17.3
	223	16	16.2		16.9	
	246			22.0		17.4

at − 55° C. In a number of instances arterial samples were also obtained for comparison.

When serial samples of blood were taken, it was necessary to withdraw only very small volumes of blood in order that the blood volume be altered as little as possible. This was done by drawing blood directly from the vein into a Roughton-Scholander (20) blood pipette (1.0 to 1.5 mm. bore, containing 100 cmm.) which was first wet with heparin solution (10 mgm. per ml.). The pipette was fitted to a 26-gauge hypodermic needle by means of a rubber gasket in the needle hub. The pressure in the vein was sufficient to fill the pipette. After removal of the needle, samples of blood for the determination of hemoglobin (20 cmm.) and specific gravity (20 cmm.) were transferred directly to appropriate pipettes, enough blood being left in the original pipette for determination of oxygen content by the method of Roughton and Scholander (20).

The results of these determinations are given in Table IX. Data for an individual animal (No. 3) are plotted in Figure 6. As swelling of the injured leg occurs, the oxygen content of the venous blood flowing from the leg increases. In some cases the venous oxygen content actually exceeded the arterial oxygen content, due to loss of plasma in passage of the blood through the injured tissue. In all animals oxygen content of both venous and arterial blood increased during the period of swelling. In three animals (Nos. 3, 13, and 7) the hemoglobin concentration was also determined in the same blood samples in which oxygen content was determined.. By using a value for oxygen capacity of hemoglobin of 1.36 ml. per gram (18), the blood oxygen saturation has been calculated for different time periods after injury. These calculations all indicate that during the period of swelling the tissue of the injured limb is utilizing oxygen (that is, tissue death did not occur from the exposure at − 55° C.). The data are not extensive enough to permit definite conclusions to be drawn as to whether oxygen utilization is decreased soon after injury. Such decrease in coefficient of oxygen utilization occurred in two of the three animals studied.

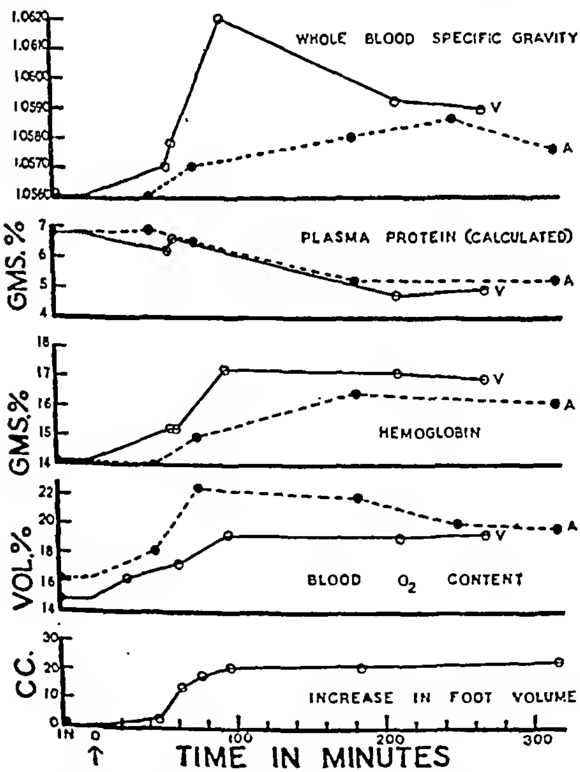

Fig. 6. Time Course of Changes in Whole Blood Specific Gravity, Plasma Protein, Hemoglobin, Blood Oxygen Content, and Foot Volume After Severe Cold Injury of One Hind Foot

Representative data from one animal after immersion of one foot at − 55° C. for 3 minutes.

Solid lines........venous whole blood or plasma

Broken lines......arterial whole blood or plasma

DISCUSSION

It has been pointed out that, following vascular occlusion of the rabbit ear prolonged enough to lead to gangrene of the part (9), the protein concentration of the edema fluid is almost identical with that occurring after frostbite. Likewise the protein concentration of edema fluid and the changes in hemoglobin and hematocrit reported here agree with those found by Harkins and Harmon (10) after freezing approximately one-quarter of the body surface of dogs. The early hemoconcentration observed to follow severe burning, which resembles that found in this series of animals following frostbite, has been observed by many workers in both animals and man (11, 12). Both the tissue edema fluid (21) and blister fluid (22) from burned areas have been demonstrated to be exudates with a protein concentration approaching that of plasma. Presman *et al.* (23) report the protein concentration of blister fluid taken from burned human beings to be about 70 per cent that of plasma. This is in good agreement with the value of 77

per cent found in this series of animals after frostbite. The plasma protein changes following burning have been studied recently by Lischer *et al.* (24). Plasma albumin tended to fall in all animals, but plasma globulin fell only in less severely burned animals which survived. It has not been determined whether the fall in plasma protein following frostbite is due chiefly to decrease in the albumin fraction.

The significance of swelling, as one of the manifestations of injury following the exposure of parts of the body to severe cold, may be judged basically from two points of view: (a) its correlation with the extent of tissue loss and the course of healing; and (b) the information it may furnish concerning the nature of the injury produced. The experiments described above indicate that the magnitude of swelling alone is of relatively little help in affording a basis for describing the final outcome of injuries produced by cold. On the other hand, the rates of its appearance and its duration have a fairly consistent relation to the severity of injury. In those animals subjected to cold of sufficient degree and duration to produce complete gangrene and loss of the exposed part, the edema which appeared was more rapid in onset and disappearance than in the milder forms of injury. In no case did gangrene appear without the previous occurrence of massive edema in the injured region.

The demonstration that the fluid lost from the blood stream into the interstitial compartment of the injured regions contained large amounts of protein furnishes evidence of increased capillary permeability. Protein was found in quantities equalling that to be expected if as much as 77 per cent of the total volume of edema fluid were whole plasma. There occurred a massive loss of protein from the blood into injured feet and ears which recovered, as well as into those which became gangrenous. Thus, injury of capillaries sufficiently severe to induce increased permeability to plasma protein does not appear to be the determining event in the development of gangrene. However, the amounts of induration and persistent periarticular fibrosis which characterize the healing stage following injury by severe cold probably depend upon the protein centent of the edema fluid.

The loss of plasma from the blood stream during the course of swelling is sufficient to produce moderate increases in hemoglobin concentration and hematocrit during the first few hours after injury. Shock was not observed in any of the animals studied, probably because of the limited size of the injury. As plasma is lost into the injured region, the blood becomes diluted by fluid from the interstitial compartment and probably from the gut as well, so that the volume of fluid lost to the frostbitten part is greater than the quantity by which the plasma volume is reduced. While the replacement of plasma volume is sufficient to produce a fall in plasma protein concentration during the first few hours after injury, it is not enough to compensate completely for the fluid loss, and a moderate increase in hematocrit occurs.

SUMMARY

Following severe cold injury, produced by immersion of one hind foot of rabbits in liquid at − 55° C. for 3 minutes, massive local edema of the part is an inevitable consequence. Investigation of the specific details involved in the development of the edema permit the following conclusions to be drawn.

1. Swelling of the foot begins as soon as thawing is complete and reaches a maximum in about 6 hours. Decrease in volume begins about 24 hours after injury and continues progressively during the next 5 days until the foot dries and becomes mummified. The maximum foot volume is about 3 times the initial volume.

2. Subcutaneous tissue pressure in frostbitten feet increases as the feet swell, until a pressure of about 25 cm. H_2O is attained 4 to 6 hours after injury at a time when the volume of the feet is maximal.

3. Fluid withdrawn from frostbitten feet during the stage of maximal edema and fluid from blisters on frostbitten ears contain an average of 4.3 per cent protein. The protein concentration of the fluid decreases progressively with time after injury.

4. The presence of toxic materials in the edema fluid was not indicated by the following tests: application to isolated guinea pig and fowl intestinal smooth muscle; intradermal injection in-

to rabbit ears; intravenous administration to rabbits while arterial pressure was being recorded.

5. The mean protein concentration of edema fluid from frostbitten feet is equivalent to about 76 per cent of the protein concentration in plasma before injury.

6. Plasma protein concentration is reduced during the first 6 hours after injury but is restored after 24 hours.

7. Moderate increases in hematocrit and hemoglobin concentration of the blood occur during the period of swelling of the foot.

8. Calculation of the extent to which fluid lost into the injured limb is replaced by extravascular fluid indicates that plasma volume is first reduced and then progressively restored during the first 6 hours after injury. Determination of plasma volume by the dye method in four animals before and after injury agrees with the plasma volume changes calculated on the basis of changes in concentration of blood constituents.

9. During the period of swelling, loss of fluid consisting of about 76 per cent plasma is rapid enough to result in decrease in plasma volume. Progressive replacement of plasma volume during the first few hours after injury is sufficient to result in a fall in plasma protein cencentration, but is not great enough to compensate completely for the fluid loss to the foot; thus, moderate increase in hematocrit occurs.

10. In some cases oxygen content of venous blood draining from the injured limb during swelling exceeded arterial oxygen content due to loss of fluid into the injured area. Oxygen utilization by the injured limb occurs during the first 5 hours after injury.

BIBLIOGRAPHY

1. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. I. A method for producing gangrene by means of controlled injury by cold. J. Clin. Invest., 1947, 26, 229.
2. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. II. General course of events following cold injury of rabbit feet and ears. J. Clin. Invest., 1947, 26, 236.
3. Henderson, Y., Adventures in Respiration. The Williams and Wilkins Co., Baltimore, Md., 1938, p. 243.
4. Wells, H. S., Youmans, J. B., and Miller, D. G., Jr., Tissue pressure (intracutaneous, subcutaneous and intramuscular) as related to venous pressure, capillary filtration and other factors. J. Clin. Invest., 1938, 17, 489.
5. Landis, E. M., The capillary blood pressure in mammalian mesentery as determined by the microinjection method. Amer. J. Physiol., 1930, 93, 353.
6. Landis, E. M., Capillary pressure and capillary permeability. Physiol. Rev., 1934, 14, 404.
7. Barbour, H. G., and Hamilton, W. F., The falling drop method for determination of specific gravity. J. Biol. Chem., 1926, 69, 625.
8. Weech, A. A., Reeves, E. B., and Goettsch, E., The relationship between specific gravity and protein content in plasma, serum, and transudate from dogs. J. Biol. Chem., 1936, 113, 167.
9. Pochin, E. E., Oedema following ischaemia in the rabbit's ear. Clin. Sci., 1942, 4, 341.
10. Harkins, H. N., and Harmon, P. H., Thermal injuries; the effects of freezing. J. Clin. Invest., 1937, 16, 213.
11. Harkins, H. N., The Treatment of Burns. Charles C. Thomas, Springfield, Ill. and Baltimore, Md., 1942.
12. Harkins, H. N., The present status of the problem of thermal burns. Physiol. Rev., 1945, 25, 531.
13. Barsoum, G. S., and Gaddum, J. H., The pharmacological estimation of adenosine and histamine in blood. J. Physiol., 1935, 85, 1.
14. Anrep, G. V., Barsoum, G. S., Talaat, M., and Wieninger, E., Further observations upon the release of histamine by skeletal muscles. J. Physiol., 1939, 96, 240.
15. Kellaway, C. H., and Rawlinson, W. A., Studies on tissue injury by heat. III. Isolated limb preparations. Austral. J. Exper. Biol. Med. Sci., 1944, 22, 83.
16. Shorr, E., Zweifach, B. W., and Furchgott, R. F., On the occurrence, sites, and modes of origin and destruction, of principles affecting the compensatory vascular mechanism in experimental shock. Science, 1945, 102, 489.
17. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B., and Archibald, R. M., Copper sulfate method for measuring specific gravities of whole blood and plasma. U. S. Navy Res. Unit, Hosp. of Rockefeller Inst. Med. Res., New York.
18. Bernhart, F. W., and Skeggs, L., The iron content of crystalline human hemoglobin. J. Biol. Chem., 1943, 147, 19.
19. Hopper, J., Tabor, H., and Winkler, A. W., Simultaneous measurements of the blood volume in man and dog by means of Evans blue dye, T-1824, and by means of CO; normal subjects. J. Clin. Invest., 1944, 23, 628.
20. Roughton, F. J. W., and Scholander, P. F., Micro gasometric estimation of the blood gases. I. Oxygen. J. Biol. Chem., 1943, 148, 541.

21. Beard, J. W., and Blalock, A., Experimental shock. VIII. The composition of the fluid that escapes from the blood stream after mild trauma to an extremity, after trauma to the intestines and after burns. Arch. Surg., 1931, 22, 617.

22. McIver, M. A., A study in extensive cutaneous burns. Ann. of Surg., 1933, 97, 670.

23. Presman, D. L., Janota, M., Weston, R. E., Levinson, S. O., and Necheles, H., Intensive human serum treatment of burn shock and a modified formula for calculating the amount of infusion. J. A. M. A., 1943, 122, 924.

24. Lischer, C., Elman, R., and Davey, H. W., Experimental burns. III. Changes in plasma albumin and globulin. War Med., 1944, 5, 43.

STUDIES ON GANGRENE FOLLOWING COLD INJURY. IV. THE USE OF FLUORESCEIN AS AN INDICATOR OF LOCAL BLOOD FLOW: DISTRIBUTION OF FLUORESCEIN IN BODY FLUIDS AFTER INTRAVENOUS INJECTION[1]

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The use of fluorescein as a means of determining the adequacy of blood flow and the degree of capillary permeability in both normal individuals and in those manifesting various pathological states has been described in a series of papers by Kurt Lange and his associates (1, 2, 3). In general, the method depends upon the introduction of fluorescein into the blood stream and its detection by exposing the region under study to ultraviolet light. Data have been obtained by direct observations, photographic recording, and photoelectric registration of the yellow-green glow which results from the excitation of fluorescein in the tissues. It is obvious that the appearance of fluorescence in tissues remote from the site of injection of fluorescein demonstrates the existence of circulating blood within the area under observation. However, the interpretation of the significance of gradations in the intensity of fluorescence, the rate at which maximum intensity is reached, and the rate at which it diminishes requires detailed knowledge of the properties of fluorescein and the manner in which it becomes distributed in blood and tissue fluid.

Changes in the time-intensity relationships of fluorescence after the intravenous injection of fluorescein have been used recently as the basis for explanations of certain abnormalities present in myxedema and in tissues subjected to severe injury by cold (4, 5). In both of these widely different abnormal states, certain of the divergences from normal in the distribution of fluorescein were attributed to changes in capillary permeability. While there is no doubt that the permeability of the capillary membranes must play an important part in determining the distribution of dye between the blood and the interstitial fluid, the influence of other factors affecting the blood-interstitial fluid equilibrium should not be overlooked. The recent important contributions of Zweifach and his coworkers (6) serve to illustrate how changes in the pattern of local blood flow may first alter, in a striking way, the freedom of exchanges between the flowing blood and the interstitial fluid and later give rise to stasis and changes in capillary permeability as secondary phenomena.

The experiments to be described below were undertaken to study in normal rabbits the distribution of intravenously injected fluorescein in blood and other body fluids, some of the factors influencing fluorescein equilibrium, and the extent of penetration of cells by the dye.

METHODS

Fluorescein was administered to normal adult rabbits by injection into the marginal ear vein. The dose used was 75 mgm. per kgm. body weight and was given as a 5 per cent solution of sodium fluorescein in distilled water. White rabbits were used in order to avoid errors due to skin pigment, and all areas for study were closely clipped. The source of the ultraviolet light used for the excitation of fluorescein was a Shannon unit No. 92 consisting of a mercury vapor spot lamp (HGCH4) mounted in a holder with adjustable trunnions on a transformer and fitted with two glass filters: a UV heat-resisting Red-Purple and a UV Blue Purple Ultra.

Photoelectric measurement of the intensity of skin fluorescence was accomplished by means of a photometer constructed from a General Electric light sensitive cell of the barrier layer type connected through a tap switch to a reflecting galvanometer. The cell was mounted in a light-shielded housing set at a distance of 10 cm. from the surface of the skin area to be studied. A metal shield, set in the plane of the skin surface, restricted the area, which served as the source of light falling on the cell, to a circle 37 mm. in diameter. The ultraviolet lamp

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

was held in a clamp with the surface of the globe at a distance of 11 cm. from the shield with the incident light directed on the skin at an angle of 45°, and at 90° to the path of the visible light from the skin surface to the light-sensitive cell. Light falling on the photo cell was restricted to the yellow-green region of the spectrum by means of a Wratten No. 61 filter mounted in the photocell housing.

The intensity of fluorescence was expressed in units of galvanometer deflection. Galvanometer readings, taken when the skin was exposed to ultraviolet light before fluorescein injection, were subtracted from those after fluorescein to yield the change due to the presence of dye. The blank reading amounted to about 5 per cent of the maximum deflection under full fluorescence in normal animals.

The concentration of fluorescein in blood and peritoneal fluid in most of the experiments was measured by adding 1 ml. of the fluid in question to 6 ml. of 95 per cent ethyl alcohol and separating the precipitated protein by centrifugation. The fluorescein dissolved freely in the alcohol used for dilution and precipitation. The resulting yellow color was measured in a Klett Summerson photoelectric colorimeter with a Wratten No. 44a filter. The concentration of the dye was calculated by the comparison of colorimeter readings with values obtained from a standard curve plotted from colorimeter readings made on alcoholic solutions of fluorescein of known concentration. Blanks were prepared from a mixture of alcohol and fluorescein-free blood to obtain a zero setting of the colorimeter. Comparison of readings from solutions containing known amounts of fluorescein dissolved in alcohol with readings obtained upon alcoholic extracts of blood to which known amounts of fluorescein had been added showed that the yield of fluorescein was complete and that no interfering pigment was retained in the extract.

A few of the analyses were carried out without extraction. Twenty cu.mm. of the blood or other fluids were diluted to 50 ml. with phosphate buffer at pH 7.0, and the concentration of fluorescein was measured in a Coleman Photofluorometer against solutions of known fluorescein concentration. Good agreement was obtained between the results from both methods of analysis.

RESULTS

1. *The relation between blood concentration of fluorescein and the fluorescence of the skin.*

The gross appearance of fluorescent skin under ultraviolet light after the intravenous injection of fluorescein indicated an initial distribution of dye in close relation to blood vessels. Within a very brief time fluorescence was uniform over the whole area in a manner consistent with the free passage of dye between the blood and the extravascular fluid. The time relations of this equilibrium were explored in normal rabbits by

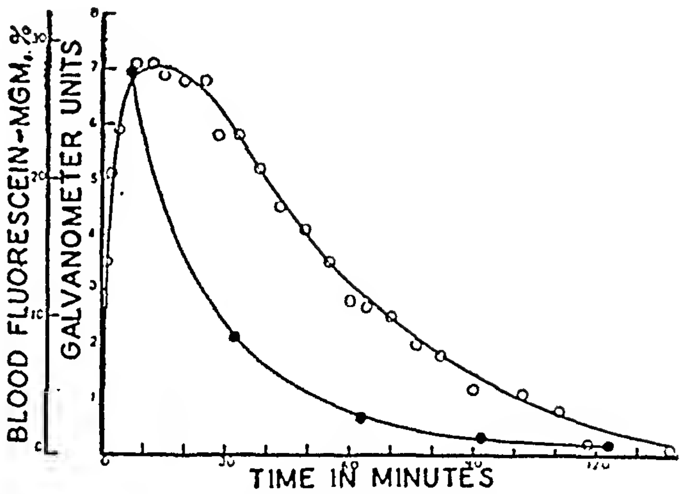

FIG. 1. CHANGE IN INTENSITY OF FLUORESCENCE IN NORMAL RABBIT'S EAR WITH TIME COMPARED TO DISAPPEARANCE OF FLUORESCEIN FROM THE BLOOD

Representative data from one animal. Open circles—fluorescence, in galvanometer units. Solid circles—concentration of blood fluorescein.

making serial measurements of both the concentration of fluorescein in the blood and measurements of the intensity of skin fluorescence. Figure 1 shows curves plotted from representative data from one animal. The curve describing the rate of disappearance of fluorescein from the blood is of the "die-away" type and indicates the dependence of rate of removal upon concentration gradients. Since fluorescein is excreted freely in the urine (3) at the same time as it is being moved from the blood stream to the extravascular fluid, this curve represents the change in concentration resulting from loss of dye as well as its dilution in the entire extracellular phase. The maximum intensity of skin fluorescence was reached at a time when the blood concentration was falling rapidly. Fluorescence declined very little over the following period of about 15 minutes. In this short interval the blood concentration of dye decreased sharply by 18 to 20 mgm. per 100 ml. In the period from 30 minutes after the injection of fluorescein up to 140 minutes, the decline of intensity of fluorescence exhibited an orderly decrease in rate with time. Thus, the curve describing decreasing fluorescence from 30 minutes after injection is qualitatively similar to that describing the change in blood concentration of dye.

The regularity of the rate of disappearance of fluorescein from the blood of rabbits is illustrated

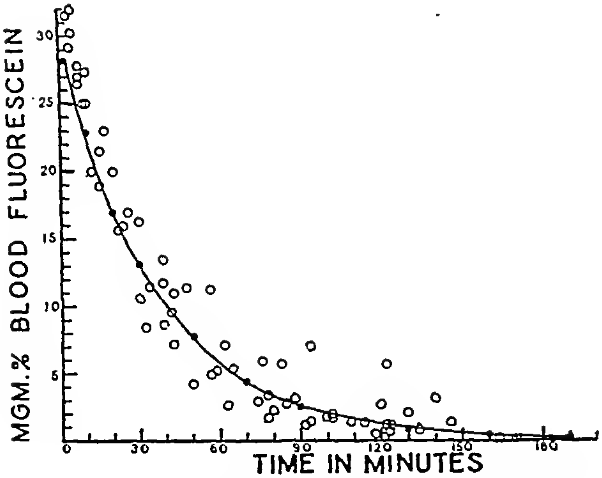

FIG. 2. CHANGE IN BLOOD FLUORESCEIN CONCENTRATION WITH TIME IN NORMAL RABBITS

Data from determinations in 14 animals after intravenous injection of 75 mgm. per kgm. sodium fluorescein. Open circles—individual determinations. Curve with solid circles fitted to data on a semi-log plot by method of least squares (Figure 3).

by the curve in Figure 2. The open circles represent individual measurements of blood fluorescein on 14 animals. The same data were plotted as the logarithm of the blood concentration against time in Figure 3. The rectilinear fit in Figure 3 was calculated by the method of least squares. The line drawn through the solid circles in Figure 2 was derived from the least square fit by the general formula

$$\log y = a + bx.$$

The constants for the line drawn in Figure 3 are: $a = 2.48$ and $b = 0.0118$.

A similar regularity in the rate of decrease of fluorescence is illustrated in Figure 4. This graph shows the distribution of the measurements on the ears of six normal rabbits of fluorescence intensity expressed as the log of the intensity in galvanometer units plotted against time. Each point represents an individual measurement. The straight line was fitted by the method of least squares. The constants are $a = 1.99$ and $b = 0.0087$. Statistical analysis of the slopes of the lines in Figures 3 and 4 showed that they are not significantly different.

These observations show that the maximum skin fluorescence does not coincide in time with maximum concentration of dye in the blood but rather occurs at a time when the blood fluorescein concentration has been sharply reduced. Thus, an important influence upon the rate of loss of dye from the blood stream is the rate at which it accumulates in the extravascular fluid, and the intensity of fluorescence in the skin must be considered an expression of extravascular fluorescein concentration rather than an expression of either blood flow rate or the concentration of fluorescein in the blood. However, it is clear that the rate at which the dye may enter or leave the extravascular fluid is dependent in some way upon concentration gradients. These data do not permit a decision as to the relative importance of diffusion or filtration as factors determining the rate of transfer.

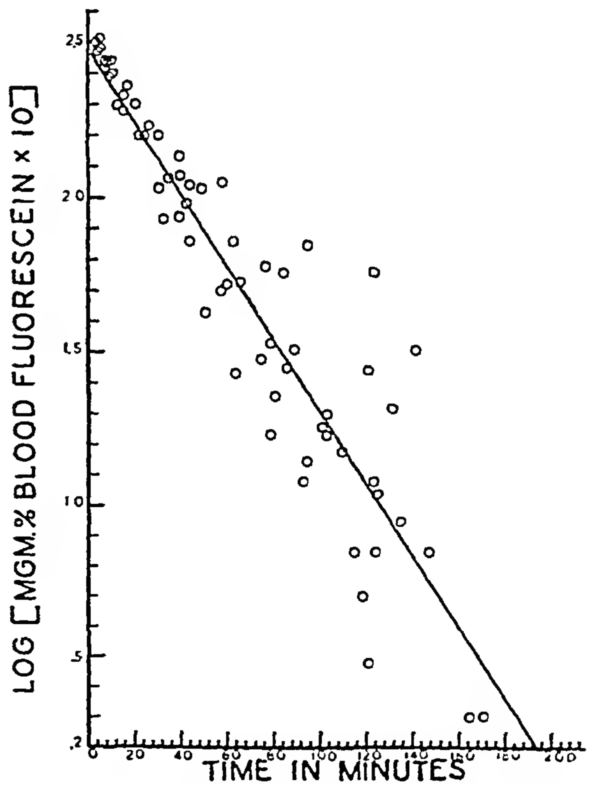

FIG. 3. CHANGE IN BLOOD FLUORESCEIN CONCENTRATION WITH TIME IN NORMAL RABBITS

The logarithm of blood fluorescein concentration is plotted as a function of time for various intervals after intravenous injection of 75 mgm. per kgm. sodium fluorescein. Straight line calculated by the method of least squares. Each point represents a single determination. Data from 14 animals.

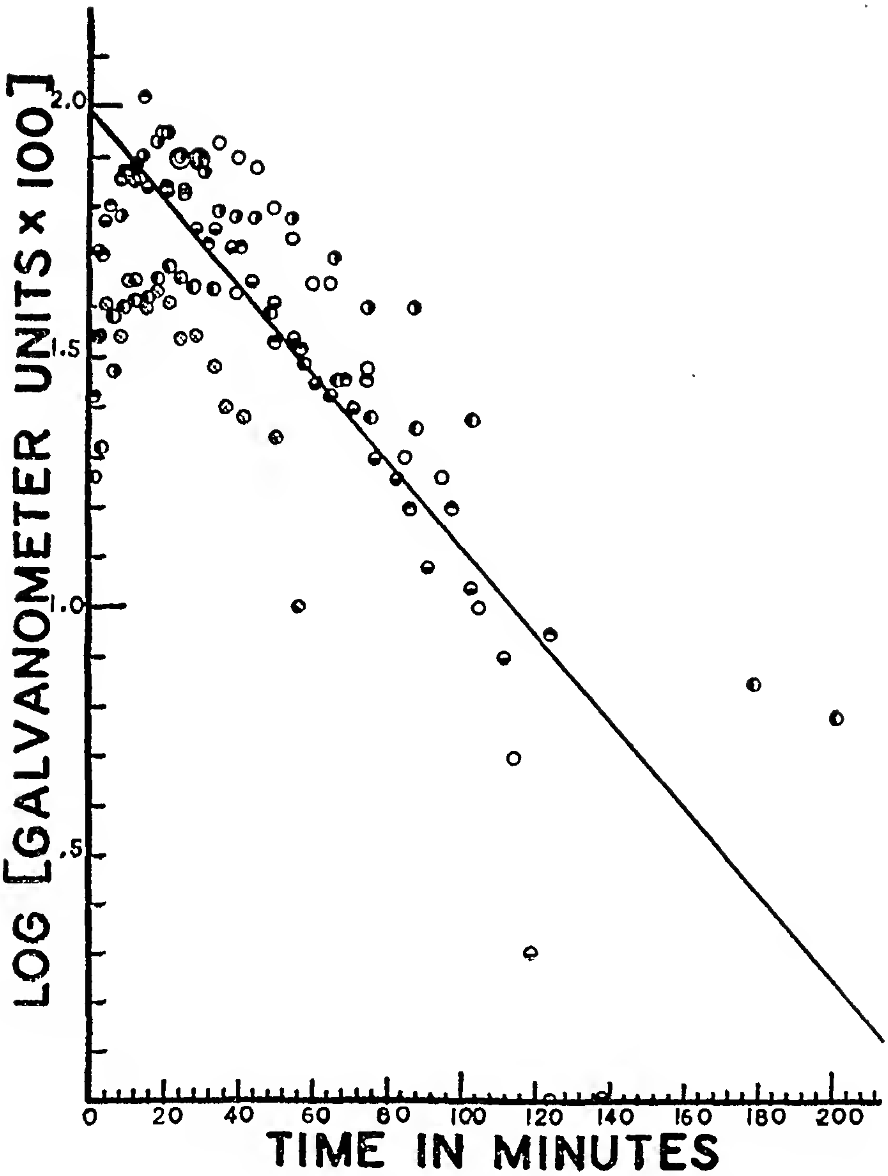

Fig. 4. Intensity of Fluorescence of Rabbits' Ears Expressed as Log Galvanometer Units

Straight line calculated by the method of least squares. Data from 6 animals. Each symbol represents 1 animal. Fluorescein injected at zero time.

2. *The diffusion of fluorescein from the blood against a filtration gradient*

Although Lange and Boyd (3) have studied the role of diffusion and filtration in exchanges of fluorescein across membranes, their experiments were carried out *in vitro* with collodion membranes. In order to demonstrate *in vivo* the free passage of dye from the blood into extravascular fluid under circumstances where the net filtration gradient was bringing about a movement of fluid into the blood stream, fluorescein exchanges were studied in three rabbits which had received intraperitoneal injections of Ringer's solution before dye was administered.

Each animal received 50 ml. per kilogram body weight of Ringer's solution containing 0.2 per cent glucose. The Ringer's solution was injected intraperitoneally, and, immediately afterward, the usual dose of fluorescein was injected intravenously. Samples of blood and of fluid aspirated from the peritoneal cavity were taken at intervals and the concentration of fluorescein was determined. After 107 to 140 minutes, the animals were killed and the volume of fluid remaining in the peritoneal cavity was measured. Estimation of the protein concentration of plasma and of peritoneal fluid was made by the method of Barbour and Hamilton (7).

TABLE I

Changes in fluorescein concentration in blood and peritoneal fluid

75 mgm. per kgm. fluorescein injected intravenously
50 ml. per kgm. Ringer's solution injected intraperitoneally

Animal number	Weight	Time of sampling. Minutes after dye injection		Plasma protein	Fluorescein	
		Blood	Peritoneal fluid		Blood	Peritoneal fluid
	kgm.			*grams per 100 ml.*	*mgm. per 100 ml.*	
23	2.20	15	12	5.78	21.5	0.70
		42	39	5.37	9.6	0.80
		85	82	5.48	2.8	1.30
		122	118	5.52	1.2	1.45
		146	148	5.34	0.7	1.40
20	2.46	10	21	4.99	27.4	0.90
		39	45	5.03	13.5	1.20
		75	82	5.41	5.8	1.40
		122	126	5.07	2.1	1.30
28	1.80	11	16	4.45	23.0	1.10
		39	43	4.66	11.8	2.40
		65	71	4.52	5.4	3.20
		102	107	4.39	1.7	2.90

TABLE II

Diffusion of fluorescein against a filtration gradient

Terminal fluid volume and fluorescein concentrations after intraperitoneal injection of 50 ml. per kgm. body weight of Ringer's solution and intravenous injection of 75 mgm. per kgm. fluorescein.

Animal number	Mgm. dye injected at 75 mgm. per kgm.	Ringer's solution			Total time	Dye in peritoneal fluid		
		Injected	Recovered	Recovery		Final conc.	Total	Injected dye
		ml.	*ml.*	*per cent*	*min.*	*mgm. per 100 ml.*	*mgm.*	*per cent*
20	181	123	53	43	126	1.3	0.69	0.38
23	165	110	72	65	148	1.4	1.10	0.67
28	135	90	50	56	107	2.9	1.45	1.07

Data obtained from three animals are presented in Tables I and II. Figure 5 shows curves describing the time course, in a representative experiment, of the changes of dye concentration in the blood and in the Ringer's solution withdrawn from the peritoneal cavity. The intersection of the curves at 110 minutes after the injection of fluorescein shows the time at which the concentration of the dye was equal in blood and peritoneal fluid. For approximately 40 minutes thereafter, the concentration of dye in blood continued to decrease while that of the fluid in the peritoneal cavity decreased comparatively little. Since the change in fluid volume in the peritoneal cavity of all three animals (Table II) showed vigorous absorption during the time that dye was passing into this fluid, the net change in fluorescein concentration was one largely determined by diffusion pressure. The permeability of capillaries within the peritoneal cavity during the experiments was assumed to undergo no significant change, since the highest specific gravity measured on peritoneal fluid did not exceed 1.0066. The data in Table I show that the concentration of dye in the peritoneal fluid reached and exceeded the concentration in the blood in two of the animals tested. In the remaining animal (No. 20), the concentration of dye in the peritoneal cavity began to decline before it reached equilibrium with the blood.

Teorell (8) has made an extensive theoretical analysis of the kinetics of distribution of substances introduced into the body on the basis of the following relationships derived from Fick's law:

(a) the total *amount* of substance transferred between blood and the tissue is:

$$\underset{-dN}{\text{amount}} = \underset{D}{\text{diffusion coeff.}} \times \underset{dc/dx}{\text{conc. gradient}} \times \underset{A}{\text{surface}} \times \underset{dt}{\text{time}} \quad (1)$$

and

(b) since the diffusion coefficient is related to the net value of the friction coefficients encountered, it will be incorporated with the effective permeation surface (A in Eq. 1) and the boundary thickness in a "permeability coefficient" as k'_n in the following:

$$\text{Amount across boundary in the time unit } \frac{-dN}{-dt} = \underset{k_n'}{\text{perm. coeff.}} \times \underset{\frac{N_i}{V_i} - \frac{N_o}{V_o}}{\text{conc. diff.}} \quad (2)$$

where N_i, V_i and N_o, V_o are the amounts of the substance and the fluid volume respectively inside and outside the boundary, and "amount" is the number of gram molecules or grams or any unit.

Where it may be assumed safely that the dis-

tribution of the substance introduced into the blood is not complicated by selective accumulation in depots, reaction with blood constituents, by tissue inactivation or by changes in volumes of fluid concerned, Teorell's analysis, by the simple formulation given above, may be applied. His calculations show that, after the concentration of substances in the blood and in the extravascular phase have become equal, the removal of substances from both phases occurs at about the same rate but that the concentration of substances in tissue fluid remains persistently above that in the blood.

The conditions of the experiments reported here differ from those in Teorell's analysis in two ways: the dye leaves the blood and enters a large amount of fluid which is continuously changing in volume, and in addition, the blood is being continuously diluted by the fluid which is being absorbed. Zweifach (9) has pointed out that transfers of fluid across capillary membranes are "transfers of fluid in bulk." It may be assumed that the transfer of fluid from the peritoneal cavity involves also the removal of at least some of the fluorescein which had previously passed into this fluid from the blood stream.

The course of events in animal No. 20 serves to emphasize the effect of fluid filtration from the peritoneal cavity to the blood upon final concentration relationships. A simplified statement of the factors determining the concentration of dye in peritoneal fluid may be presented as follows:

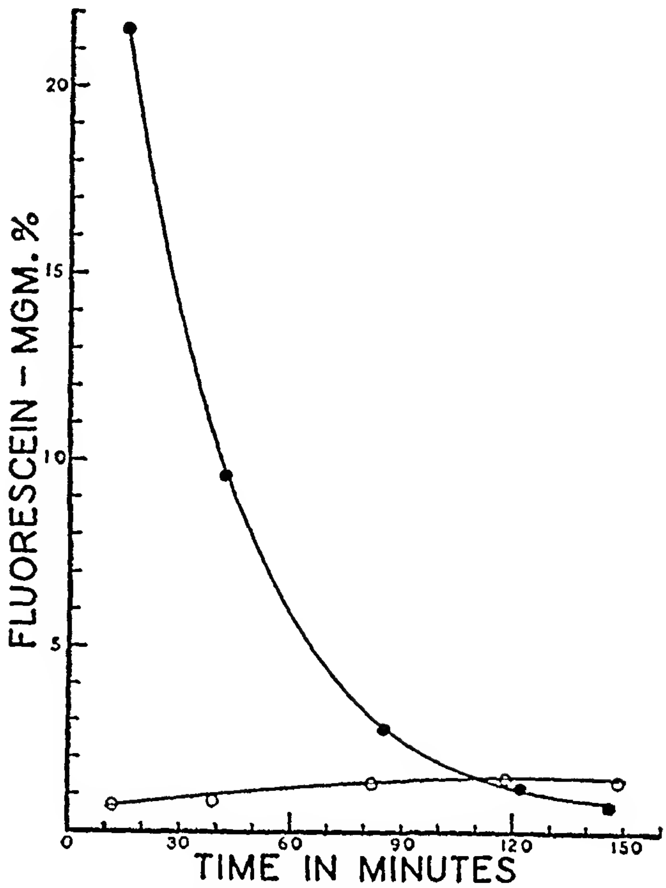

FIG. 5. TIME COURSE OF CHANGES IN FLUORESCEIN CONCENTRATION IN BLOOD AND PERITONEAL FLUID

Representative data from 1 animal. Fluorescein injected at zero time. Solid circles—concentration of fluorescein in blood. Open circles—concentration of fluorescein in peritoneal fluid.

$$\frac{(\text{dye entering mgm. per min.}) - (\text{dye leaving mgm. per min.})}{(\text{fluid entering ml. per min.} - (\text{fluid leaving ml. per min.})} = \frac{\text{mgm. gain or loss of dye}}{\text{ml. gain or loss of volume}} = \text{net change}$$

in conc. of dye and the concentration of dye in the peritoneal fluid may be expressed as

$$\text{mgm. of dye per 100 ml.} = \frac{\text{net change in conc. of dye}}{\text{volume of fluid in the peritoneal cavity}} \times 100.$$

Where the rate of fluid filtration from the peritoneal cavity is high, as it was in the case of animal No. 20, the rate of removal of dye along with the fluid may be sufficiently great to bring about a decline of fluorescein concentration in the peritoneal cavity before concentration equilibrium is established. We have no reason to believe that there was any qualitative difference in the processes involved in fluorescein exchange among the three animals tested in the manner described. The attainment of fluorescein equilibrium between blood and the Ringer's solution in the peritoneal cavity may not safely be considered to represent simple relationships of diffusion pressure and permeability. The magnitude of the equilibrium concentration and the time at which this concentration was reached depends upon the rate and direction of fluid exchange as well as upon the rate and direction of transfer of dye.

These observations show that, while fluorescein may diffuse out of the blood into extravascular fluid at the same time as fluid is being actively

taken into the blood in the same region, the movement of dye is not independent of the movement of water.

3. *The extent of cell penetration by fluorescein.*

A further examination of the distribution of fluorescein after intravenous injection was carried out by the analysis of blood and skeletal muscle from animals subjected to previous ligation of the renal pedicles. Three rabbits were anesthetized by the intraperitoneal injection of dial, 90 mgm. per kgm. body weight. After supplementary administration of ether the renal pedicles were ligated acutely either through lumbar incisions or by an abdominal approach. The wounds were closed and control blood samples were taken. An intravenous injection of fluorescein, 75 mgm. per kgm. body weight, was given. At intervals approximately one-half hour apart, three blood samples were taken. Following the last sample, the animals were killed and samples of skeletal muscle were excised. Plasma was analyzed for fluorescein by the methods described previously. The muscle samples were weighed, homogenized in a Waring Blendor and subjected to extraction for the determination of fluorescein. The muscle from the first animal studied was extracted directly with 95 per cent alcohol in the proportion of 1 gram of minced muscle to 6 ml. of alcohol. Although the muscle was grossly yellow in appearance this method of extraction yielded no detectable fluorescein when the alcoholic extract was subjected to colorimetric comparison. In the remaining two animals the muscle samples weighing approximately 50 grams each were extracted directly in 200 ml. of cold phosphate buffer at pH 7.0 or were subjected to repeated freezing and thawing before final separation of the extracting medium by centrifugation. The shapes of the curves describing the "dilution" of injected fluorescein indicated that even at the end of 150 minutes the blood concentration of the dye continued to fall slowly. Calculation of the volume of fluid in which the fluorescein would have to be dissolved, if solution alone were to account for the reduced concentration with time, showed that the apparent distribution volume of dye amounted to 39 to 45 per cent of the animal's body weight. Various investigators (10, 11) have found that the extracellular phase of most mammals including rabbits comprises between 20 and 30 per cent of the body weight. Therefore, the apparent distribution volume of fluorescein observed in the present studies must be accounted for by an appreciable penetration into the cellular compartment, or loss by some other route.

The analyses of muscle samples from the same animals yielded values for fluorescein concentration of 1.03 to 1.92 mgm. of dye per 100 grams of fresh muscle. On the assumption that even distribution of fluorescein existed throughout the extracellular phase, the comparison of plasma fluorescein with muscle fluorescein showed that the distribution volume of dye amounted to 8.4 to 11.4 per cent of the muscle weight. Others have reported that the extracellular phase of rabbit muscle is approximately 16 per cent by weight (10). It, therefore, seemed unlikely that the large total distribution volume of dye could be accounted for on the basis of a general penetration of dye into cells.

All of the animals were observed to have brightly fluorescent yellow dye in the gallbladder bile and in the lumen of the intestine. Analysis of the intestinal fluid for fluorescein in one animal showed that the concentration of dye was 45.75 mgm. per 100 ml. The apparent dilution volume of dye, estimated from the amount injected and the final concentration, was greatly in excess of the extracellular phase volume calculated as 25 per cent of the body weight; 925 ml. compared to 500 ml. The amount of dye which would be dissolved in the volume representing the difference between the two values given above at the final blood concentration of 16.25 mgm. per 100 ml. was

$$425 \text{ ml.} \times \frac{16.25 \text{ mgm.}}{100 \text{ ml.}} = 69.1 \text{ mgm.}$$

If all of this "lost dye" were in the intestinal tract at a concentration of 45.75 mgm. per 100 ml., the volume of fluid necessary to accommodate it would be:

$$\frac{69.1 \text{ mgm.}}{V} = \frac{45.75 \text{ mgm.}}{100 \text{ ml.}}$$
$$V = 151 \text{ ml.}$$

Since the brilliant fluorescence of gallbladder bile

indicated a concentration of dye even greater than that in the fluid in the lumen of the intestine, a dilution volume much less than the estimated volume of 151 ml. would actually accommodate the amount of "lost dye" indicated above. Carmichael, Strickland, and Driver (12) report data from which the water in the contents of various parts of the gastro-intestinal tract of rabbits may be calculated. The average water content of the small intestine, cecum and colon from six rabbits, weighing approximately 2 kilograms each, was 114 ml. Thus, it is reasonable to assume that the disparity between the measured distribution volume of fluorescein and that predicted for the extracellular phase may be accounted for by the amount of dye excreted into the bile and the lumen of the intestine.

The distribution of fluorescein between plasma and red blood cells was studied on blood samples from four rabbits by equilibrating heparinized whole blood *in vitro* at 37° C. with known amounts of fluorescein. The fluorescein concentration of the separated plasma was determined at intervals, and the distribution of dye between the cells and the plasma phase was calculated from these measurements and the hematocrit values. Within one minute after the addition of dye to the blood, only about 85 per cent of it could be found in the plasma, and over a period of 2 hours the plasma fluorescein diminished to 82.7 per cent of the amount predicted from the quantity of dye originally added to the sample. Thus, either penetration of fluorescein or its adsorption upon the surface of red blood cells removed from 15 to 17.3 per cent of it from the plasma.

When red cells were subjected to alteration of their normal surface volume relationship by suspending them in solutions of sodium chloride varying in concentration from 1.8 per cent to 0.54 per cent before equilibrating them with fluorescein, no differences were noted in the amount of dye recovered from the non-cellular phase of the suspension. Since there is little or no change in cell surface area and large change in cell volume under the above conditions, it seems likely that the disappearance of fluorescein from the plasma phase may be ascribed to surface adsorption rather than cell penetration.

CONCLUSION

1. The intensity of fluorescence of regions exposed to ultraviolet light after the intravenous injection of fluorescein depends more directly upon the amount of dye in the interstitial fluid than upon the concentration of dye in the blood.

2. The amount of fluorescein present in the interstitial fluid depends to an important degree upon filtration processes involved in the exchanges of plasma ultrafiltrate across capillary membranes as well as upon simple diffusion exchanges. The movement of dye across capillary membranes should not, therefore, be considered to be independent of the movement of water.

3. The distribution of fluorescein in the various water compartments of the body after intravenous injection extends throughout the extracellular phase. The dye is excreted in large amounts in the bile and small quantities, 15 to 17 per cent, become associated with the cellular fraction of the blood in a manner which suggests surface occlusion rather than penetration of the cells.

SUMMARY

The distribution of injected fluorescein, a fluorescent dye, was studied in normal rabbits. A consideration of the relation of dye concentration in the blood and the intensity of fluoresence of the animal's skin under ultraviolet light indicated that maximum intensity of fluorescence is reached at a time when the concentration in the blood is falling rapidly. Thereafter, the disappearance of dye from the blood and the decline of intensity of skin fluorescence occurred at about the same rate.

The exchanges of fluorescein injected into the blood stream with Ringer's solution introduced into the peritoneal cavity of rabbits showed that the concentration of dye rose in the fluid within the peritoneal cavity during the time that the fluid was being actively absorbed into the blood. The concentration of dye in the fluid within the peritoneal cavity at any time after injection depends upon the amount of it entering and leaving the peritoneal cavity as well as upon the direction and volume of fluid exchange. Therefore, in circumstances where the volume of extravascular fluid may be changing, the movement

of dye is not independent of the movement of water.

The distribution of intravenously injected fluorescein was tested in rabbits after ligation of the renal pedicles. Calculations of the apparent dilution volume of the dye yielded values equivalent to from 39 to 45 per cent of the animals' body weight. Determinations of the fluorescein content of samples of skeletal muscles from the same animals showed no evidence that the dye penetrated the intracellular phase. If the amounts of dye in muscle were assumed to be in equilibrium with the fluorescein in the blood, the volume of muscle water in which the dye was dissolved equalled 8.4 to 11.4 per cent of the total weight of muscle. Sufficient amounts of fluorescein were found in the bile and in the lumen of the gut to approximate the amount which had disappeared from the extracellular phase.

When heparinized whole blood was equilibrated for 2 hours *in vitro* at 38° C. with known amounts of fluorescein, approximately 83 per cent of the amount of added dye could be recovered from the separated plasma. Since osmotic manipulation of red cell volume without change in the surface area did not change the amount of dye recovered from the fluid phase, the loss of dye was tentatively attributed to surface adsorption rather than cell penetration.

BIBLIOGRAPHY

1. Lange, K., and Boyd, L. J., The use of fluorescein to determine the adequacy of the circulation. M. Clin. North America, 1942, 26, 934.
2. Lange, K., and Boyd, L. J., The technique of the fluorescein test to determine the adequacy of circulation in peripheral vascular diseases, the circulation time and capillary permeability. Bull. N. Y. Med. Coll., 1943, 6, 78.
3. Lange, K., and Boyd, L. J., Use of fluorescein method in establishment of diagnosis and prognosis of peripheral vascular diseases. Arch. Int. Med., 1944, 74, 175.
4. Lange, K., Capillary permeability in myxedema. Am. J. M. Sc., 1944, 208, 5.
5. Lange, K., and Boyd, L. J., The functional pathology of experimental frostbite and the prevention of subsequent gangrene. Surg., Gynec. & Obstet., 1945, 80, 346.
6. Zweifach, B. W., Lee, R. E., Hyman, C., and Chambers, R., Omental circulation in morphinized dogs subjected to graded hemorrhage. Ann. Surg., 1944, 120, 232.
7. Barbour, H. G., and Hamilton, W. F., The falling drop method for determining specific gravity. J. Biol. Chem., 1926, 69, 625.
8. Teorell, T., Kinetics of distribution of substances administered to the body. Arch. Internat. de Pharmacodyn. et de Thérap., 1937, 57, 205.
9. Zweifach, B. W., The structural basis of permeability and other functions of blood capillaries. Cold Spring Harbor Symp. Quant. Biol., 1940, 8, 216.
10. Harrison, H. E., Darrow, D. C., and Yannet, H., The total electrolyte content of animals and its probable relation to the distribution of body water. J. Biol. Chem., 1936, 113, 515.
11. Winkler, A. W., Elkinton, J. R., and Eisenman, A. J., Comparison of sulfocyanate with radioactive chloride and sodium in the measurement of extracellular fluid. Am. J. Physiol., 1943, 139, 239.
12. Carmichael, E. B., Strickland, J. T., and Driver, R. L., The contents of the stomach, small intestine, cecum and colon of normal and fasting rabbits. Am. J. Physiol., 1945, 143, 562.

STUDIES ON GANGRENE FOLLOWING COLD INJURY. V. THE USE OF FLUORESCEIN AS AN INDICATOR OF LOCAL BLOOD FLOW: FLUORESCEIN TESTS IN EXPERIMENTAL FROSTBITE [1]

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The application of the fluorescein test to the study of frostbite has been described recently by Lange and Boyd (1). In the course of studies of experimental frostbite carried on in this laboratory, certain of the results obtained with the fluorescein test have been difficult to explain on the basis of interpretations suggested by the proposers of the method. Two groups of experiments were undertaken in order to study certain aspects of fluorescein exchange not already reported in the literature. The first group of experiments consisted of studies on normal rabbits of skin fluorescence and fluorescein distribution in body fluids: these have been presented previously (2). The present paper reports the second group of studies carried out on normal rabbits and on rabbits subjected to severe cold injury.

METHOD

The techniques employed in the fluorescein test, the measurement of skin fluorescence after the injection of fluorescein, and the analysis of body fluids for fluorescein were described previously (2). Skin fluorescence under ultraviolet light was photographed with a Kodak Recomar having a Kodak anastigmat lens, f. 4.5 of 105 mm. focal length. Black and white photographs were taken at f.8 on Ansco Superpan Press film with exposure times of 15 seconds through a Wratten No. 15 filter or 90 seconds through a Wratten No. 62 filter. The animals used in all of the experiments were adult New Zealand white rabbits from a uniform strain.

RESULTS

1. *Fluorescence in normal skin*

When the intravenous injection of fluorescein was made while the animals were exposed to ultraviolet light, bright greenish-yellow fluorescence was detectable in hair-free areas of the skin within a few seconds after the beginning of injection. The intensity of color became maximal in about 15 minutes. The decline of intensity was, at first, rapid and then progressively more gradual over a period of 2 to 2½ hours. In the rabbit ear, the regions which glowed most intensely were those relatively free of large blood vessels. The larger blood vessels stood out as dark streaks which could be obliterated by prescure with a glass slide. The areas rendered ischemic by pressure showed no decrease of fluorescence.

2. *Fluorescence in frostbitten ears of rabbits*

The influence of injury by frostbite upon the distribution of fluorescein after intravenous injection was studied on rabbits' ears. Frostbite was produced by immersing the distal 3 to 4 cm. of the ears in a freezing mixture consisting of alcohol, ethylene glycol, and water with sufficient solid carbon dioxide added to bring the temperature between − 52° C. and − 70° C. The details of the method of producing standard cold injury and the general course of changes following such injuries have been described in previous reports (3, 4).

Abnormalities in the distribution of fluorescein in the frostbitten portion of rabbits' ears included decreased rate of entrance of dye, slower rate of removal, and lower maximum intensity of fluorescence than was observed in the normal ear. Observations made at varying intervals after injury showed that the local changes, which were responsible for the abnormalities of fluorescein distribution, undergo modification with time. The photographs in Figure 1 illustrate the delayed penetration and exit of the dye as well as the differences in results of identical tests made immediately after inquiry and repeated

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

some 18 hours later. Figure 1A shows a photograph of the normal ear of a rabbit taken under ultraviolet light before the injection of fluorescein and before the ear was frostbitten. The small bright spot near the tip of the ear is the fluorescence from a drop of lubricating oil which was smeared on the skin from the electric clipper. Figure 1B was taken 15 minutes after the ear was re-

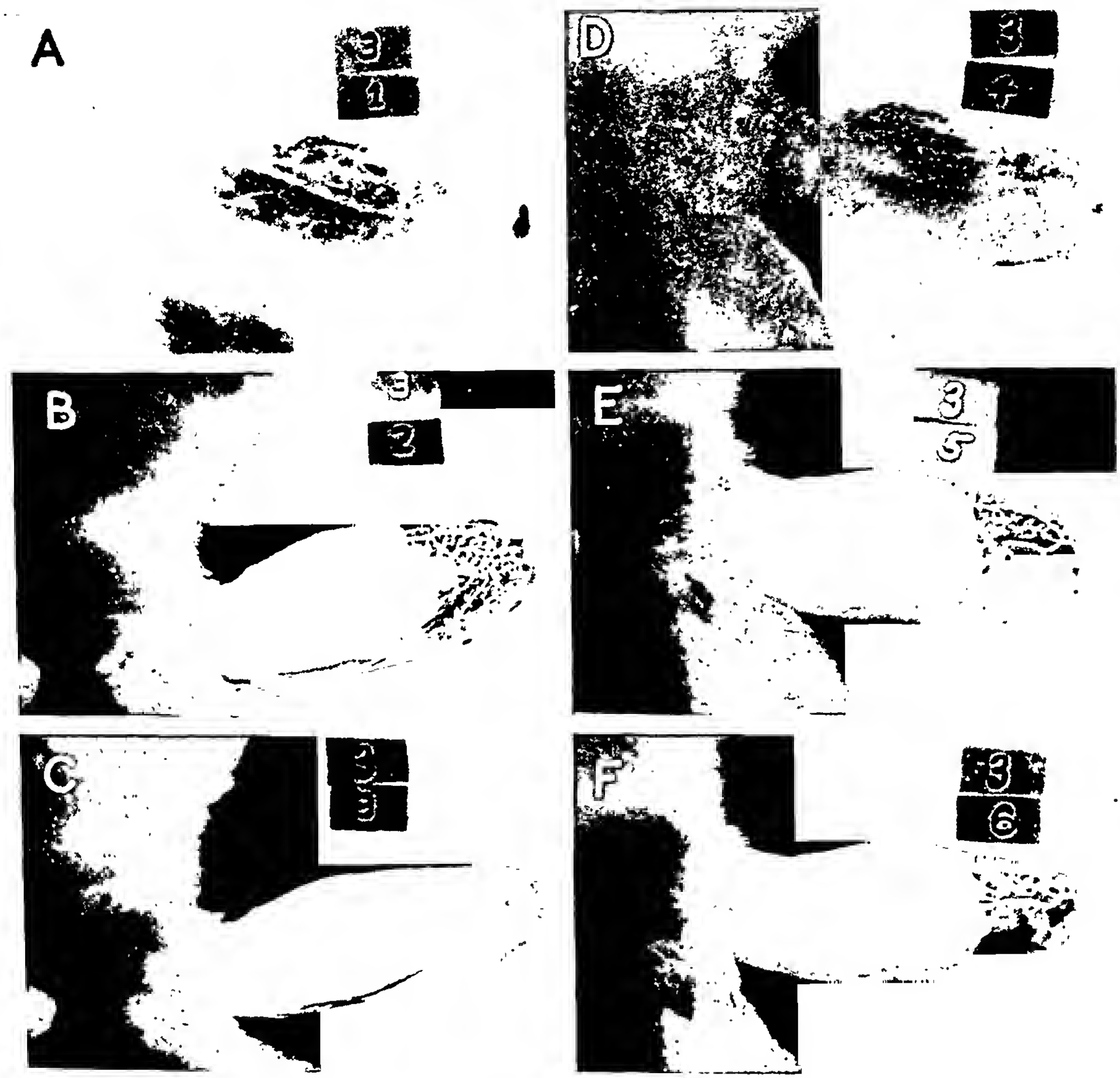

Fig. 1. Distribution of Fluorescein in a Frostbitten Rabbit Ear at Varying Times after Injury and after Fluorescein Injection

A. Normal ear under ultraviolet light before frostbite, and before injection of fluorescein.
B. 15 minutes after frostbite (1-minute immersion at − 70° C.) and 10 minutes after injection of 75 mgm. per kgm. sodium fluorescein into the marginal ear vein of the opposite ear.
C. 30 minutes after frostbite and 25 minutes after fluorescein.
D. 18 hours 30 minutes after frostbite. Fluorescein completely removed.
E. 18 hours 50 minutes after frostbite and 10 minutes after a second intravenous injection of fluorescein.
F. 19 hours 5 minutes after frostbite and 25 minutes after the second fluorescein injection.

Photographs taken with ultraviolet light from an HGCH4 lamp. Lamp filters consisted of a heat resisting red-purple and a UV blue-purple glass filter. Lens filter was Wratten No 15. Exposure 15 seconds at f.8 on Ansco Superpan Press film.

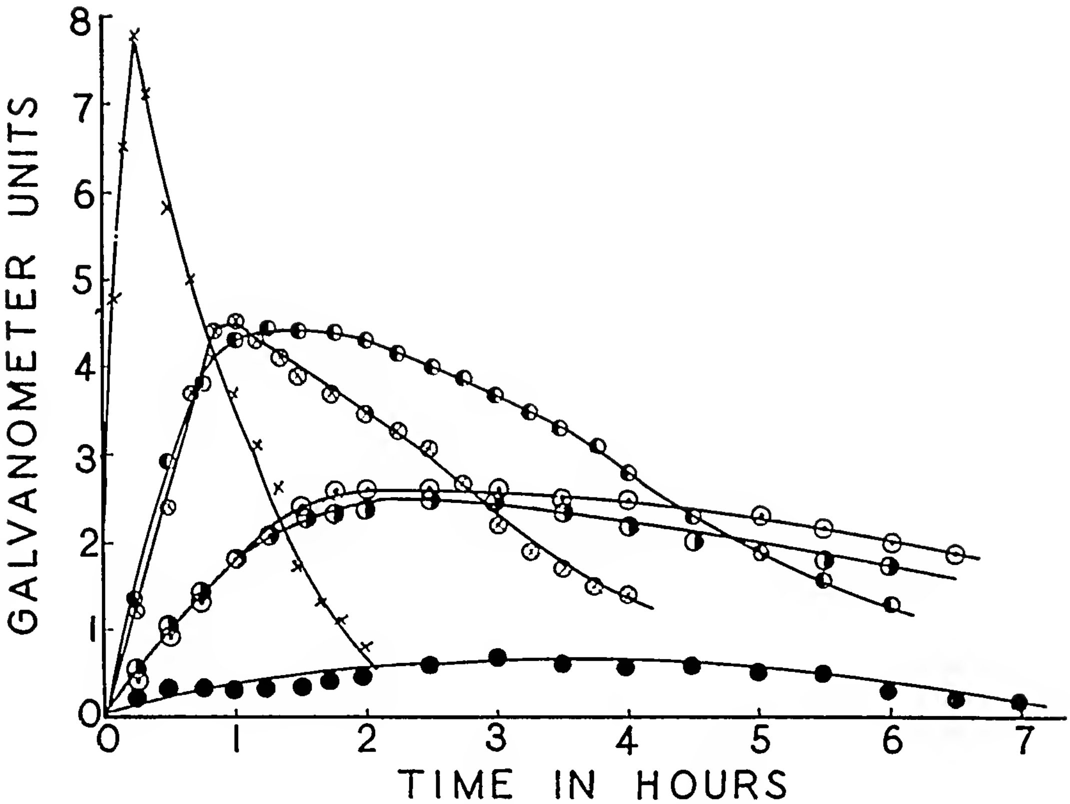

FIG. 2. INTENSITY OF FLUORESCENCE OF RABBITS' EARS AT DIFFERENT TIME PERIODS AFTER FROSTBITE

Ears exposed at − 55° C. for 90 seconds. Fluorescein injected at zero time.

× Normal ears (6 animals)
Immediately after frostbite (5 animals)
◐ 1 hour after frostbite (4 animals)
1 day after frostbite (3 animals)
◑ 2 days after frostbite (3 animals)
3 days after frostbite (2 animals)

moved from the freezing mixture. The exposure to cold was 1 minute at − 70° C.[2] Fluorescein was injected intravenously in the opposite ear 10 minutes before the photograph was taken. The depth of immersion in the freezing mixture is clearly indicated by the virtual absence of fluorescence in the distal 3 cm. of the ear. The larger vessels of the distal part of the ear are faintly fluorescent at this time, with an appreciable spread of dye about the central artery for a distance of about 8 mm. into the injured area. Some lack of sharpness in the junction between the brightly fluorescent, normal part and the injured region suggests that limited penetration of fluorescein was taking place at that point. Figure 1C is a photograph of the same animal taken 30 minutes after frostbite and 25 minutes after the injection of dye. It will be noted that the intensity of fluorescence had diminished to a considerable degree in the normal part of the ear, while that in the injured area had reached a brightness almost equal to that observed at 10 minutes in the uninjured areas, and that the fluorescence almost obscures vascular detail. Figure 1D, photographed 18½ hours after the first injection of fluorescein, shows the complete removal of dye from all parts of the ear at that time. Figure 1E illustrates the appearance of the ear at 18 hours and 50 minutes after frostbite and 10 minutes after the second injection of fluorescein. In this photograph the vascular detail, which was easily made out in the normal part of the ear at 10 minutes after the first injection of dye, is almost completely obscured by the massive edema and the distribution of fluorescein in the skin. The penetration of dye into the injured region can be seen only faintly outlining the major vascular

[2] The results were the same when the temperature of the freezing mixture was − 52° to − 56° C.

channels. Figure 1F, photographed 25 minutes after the second injection of fluorescein, shows a striking reduction in the amount of dye in the injured region as compared with the amount which accumulated during an identical interval after injection when the injury was only 30 minutes old.

3. *Time intensity relationships of fluorescence in normal and frostbitten ears of rabbits*

Photoelectric measurements were made of the change in intensity with time of fluorescence in 6 normal rabbit ears and in 17 ears at various times after frostbite. Figure 2 shows the results of these measurements in curves plotted with the intensity of fluorescence on the axis of ordinates in terms of galvanometer units and time in hours after the injection of fluorescein on the axis of abscissas. In normal ears, the change in itensity of fluorescence with time was characterized by a very rapid rise to a maximum within 15 minutes and a somewhat less rapid decline in intensity, with final disappearance of glow occurring between 2 and 3 hours after injection of dye. The rate of increase in intensity measured in frostbitten ears when the dye was injected immediately or at 1 hour after injury is slower than that observed in normal ears. When the injection of fluorescein was made at successively longer intervals after frostbite, further slowing of both the entrance and exit of dye occurred. At intervals of 1 and 2 days after frostbite the maximum intensity reached was only a little over a third of that reached in normal ears. The tendency for exit of fluorescein from the frostbitten region to be slower than its entrance, a phenomenon noticeable in the comparison between measurements made when the dye was injected immediately after frostbite with those made at 1 hour, was greatly exaggerated in the tests made at intervals of 1 and 2 days. While no striking differences were noticeable between the measurements made on the first and second days after injury, the tests on the third day showed only a feeble fluorescence. The maximum occurred about 2 hours after the injection of dye and was followed by a greatly prolonged course of removal. In some cases fluorescence was detected by direct observation as long as 24 hours after dye injection.

Since the rates of entrance and removal of dye were shown to become progressively slower as the interval between injury and the test was increased, only the earliest tests were subjected to statistical comparison. The significance of difference in rates of fluorescein exchange between that in normal ears and that in ears tested immediately after frostbite was calculated (5). Table I presents measurements of fluorescence at 10 minutes after the injection of dye and at 2 hours after injection in 5 normal ears and in 5 frostbitten ears of rabbits receiving fluorescein immediately after frostbite. Comparison of the intensity of fluorescence in both series at 10 minutes after injection shows that the brilliance is significantly greater in normal ears. Analysis at this time interval was considered to be free of differences which might be attributed to gross deficiencies of blood flow in the frostbitten ears because skin temperature measurements taken at this time showed that the temperature was rising very rapidly. The pink color of the skin and the large size of major blood

TABLE I

Measurements of intensity of fluorescence in normal and frostbitten ears of rabbits at intervals after injection of fluorescein

Data from 5 animals

Column	Normal ears				Frostbitten ears 90-second exposure at −55° C.			
	1	2	3	4	5	6	7	8
	Fluorescence in galvanometer units			Time to maximum	Fluorescence in galvanometer units			Time to maximum
	10 min.	Maximum	Decr. 2 hrs.*		10 min.	Maximum	Decr. 2 hrs.*	
				min.				*min.*
	8.5	10.4	9.5	15	0.6	7.5	2.7	70
	7.5	9.0	9.0	19	1.5	5.5	2.0	58
	4.5	4.5	4.5	10	1.5	4.2	0.8	54
	7.1	7.1	7.0	9	0.3	3.5	2.5	60
	4.0	4.8	3.7	20	0.2	2.0	1.5	84
$\bar{X}$ =	6.78		6.74		0.82		1.90	
$SE_{\bar{x}}$ =	0.752		1.163		0.285		0.344	

$\bar{X}$ = Mean.

$SE_{\bar{x}}$ = Standard error of mean.

* Difference in galvanometer units between maximum readings and readings taken two hours after injection of fluorescein. Comparison of mean values at the base of columns 1 and 5 indicates difference in rate of entrance of dye in normal and frostbitten ears. Standard error of the difference between means of columns 1 and 5 is 1.213 and $P < 0.01$ Comparison of columns 3 and 7 indicates difference in rate of exit of the dye Standard error of the difference between the two means is 0.804 and $P < 0.001$. P expresses the probability that such differences would be encountered by errors of random sampling.

vessels also furnished evidence of rapid blood flow.

The fluorescence at 10 minutes, at the maximum, and the decrease in 2 hours following the injection of dye (see Table I) was greater in normal ears than in those injured by cold. Statistical comparison of the mean values for columns 1 and 5 showed a significant difference. Therefore, since the most rapid rate of entry of dye into frostbitten ears is significantly slower than the rate of entry into normal ears, the still slower rates of entry encountered at longer intervals after injury must also be lower than the rate in normal ears. The slowing of removal of dye from frostbitten ears as compared with the rates of removal from normal ears was found to be significant by means of a similar assessment of the difference between the means of values given in columns 3 and 7. The data in columns 3 and 7 were obtained by subtracting from the galvanometer deflection measured at the peak of fluorescence, the galvanometer deflection measured 2 hours after the maximum was reached. The difference between these amounts of change over a period of 2 hours in the two groups is consistent with the marked difference in the general slopes of the curves of declining intensity of fluorescence. Progressive flattening of the curves is noted with increase in the interval between injury by cold and the injection of dye. Hence, the validity of the difference established between measurements on normal ears and on frostbitten ears tested immediately after frostbite may be presumed to extend to the greater differences observed at longer time intervals.

TABLE II

Comparison of fluorescein concentration in blood and edema fluid

75 mgm. per kgm. fluorescein given intravenously 10 minutes after exposure of one foot at −55° C. for 3 minutes

Animal number	Time after fluorescein	Blood fluorescein concentration	Plasma fluorescein* concentration	Edema fluid fluorescein concentration
	min.	*mgm. per 100 ml.*	*mgm. per 100 ml.*	*mgm. per 100 ml.*
78	120	1.78	2.92	5.10
74	140	3.20	5.25	10.78
81	175	0.20	0.33	8.25

* Plasma fluorescein concentration calculated from whole blood fluorescein concentration assuming a hematocrit of 39 per cent (mean of 9 animals) and no penetration of fluorescein into red blood cells.

4. *The concentration of fluorescein in edema fluid and in blood*

In order to determine whether the delayed removal of fluorescein from frostbitten regions was a phenomenon restricted to layers of the skin near the surface or one involving the deeper layers also, analyses of blood and of edema fluid from frostbitten feet were made in three animals. One hind foot of each of three rabbits was frostbitten by immersion for 3 minutes in the freezing mixture at − 55° C. (3). Intravenous injection of fluorescein, 75 mgm. per kgm. body weight, was given at 10 minutes after frostbite. At intervals from 120 to 175 minutes after the injection of dye, samples of blood and of edema fluid were taken for fluorescein analysis. Data from these experiments are presented in Table II. The low concentrations of dye found in the blood of these animals is similar to that observed in normal animals at comparable times after fluorescein administration (2). Edema fluid, withdrawn from the frostbitten feet at the same time as the blood samples were obtained, contained dye at several times the concentration found in the blood. On the basis of an estimated hematocrit value of 39 per cent in rabbit No. 78, where the smallest difference is shown, the calculated plasma concentration of dye was 2.92 mgm. per 100 ml., only a little more than half of that in the edema fluid from the foot of the same animal.

5. *The influence of increased blood flow upon fluorescein exchanges in frostbitten rabbit ears*

Measurements of skin temperature in frostbitten ears and feet of rabbits have shown that the blood vessels of the injured regions do not participate in thermovascular reflex changes in diameter (4). However, the vessels of parts of ears proximal to the injured region show both tonic constriction and the ability to dilate in response to local application of heat or following procaine block of the stellate ganglions (6). The flow of blood into the injured area of frostbitten ears is, in part, determined by the degree of dilatation of the major arteries proximal to the line of frostbite. Direct observation of blood flow in frostbitten ears

treated by rapid thawing in warm water or by procaine block of the stellate ganglion showed that the irreversible stage of stasis was delayed when blood flow was increased, whether the increase was brought about by heat or by sympathetic paralysis (6). If stasis was allowed to progress without treatment for 20 to 50 minutes, the subsequent increase of blood flow was ineffective in restoring movement of blood in true capillaries as determined by direct observation.

The influence of increased blood flow upon the distribution of fluorescein in the frostbitten ears of rabbits was tested in the following manner. Both ears of each of three rabbits were frostbitten by immersion for 1 minute in the freezing mixture at − 55° C. At intervals varying from 54 to 125 minutes after frostbite, 1 to 2 ml. of 2 per cent procaine hydrochloride solution were injected into the region of the stellate ganglion on one side. Five minutes later fluorescein was injected by vein in the usual dose of 75 mgm. per kgm. body weight. Over the ensuing 2 to 6 hours, the intensity of fluorescence was measured in both ears. The curves presented in Figure 3 show that in all three animals the fluorescence of the ear on the side subjected to sympathetic block reached a higher maximum intensity than that measured on the injured, untreated ear. While the rate of increase in fluorescence was higher on the blocked side, the rate of removal was about the same for both.

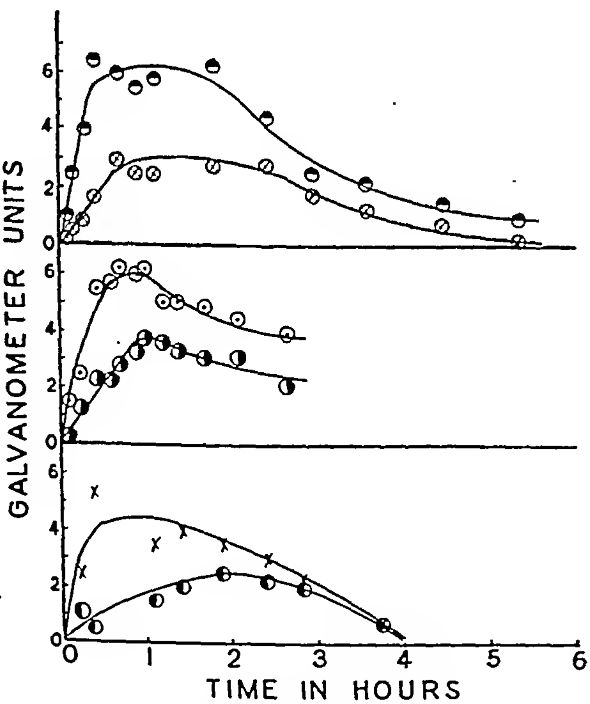

FIG. 3. INTENSITY OF FLUORESCENCE OF RABBITS' EARS AFTER FROSTBITE AND PROCAINE BLOCK OF RIGHT STELLATE GANGLION

Both ears of each rabbit exposed at − 55° C. for one minute. Procaine injected 2 hours after frostbite. Fluorescein injected .5 minutes after procaine (zero time on figure). Upper curves in each figure—right frostbitten ear, ganglion blocked. Lower curves in each figure—left frostbitten ear. Each figure represents one animal.

The increase in fluorescence, observed in frostbitten ears under the circumstances described above, was measured at a time after injury when stasis in true capillaries was completely developed. Paralysis of the sympathetic nerve supply to the ear, at comparable times after injury in other experiments, had been found to be incapable of restoring blood flow in these vessels (6), even though the rise in skin temperature indicated an increased total blood flow. Since the exposure to cold of both ears in each animal was the same, the difference in intensity of fluorescence following procaine block of the stellate ganglion should be attributed to alteration of the minute volume or local pattern of blood flow rather than to differences in capillary permeability.

DISCUSSION

Observations of skin color, skin temperature, arterial pulsation, and movement of blood watched under the microscope, at least in some vascular channels, in the frostbitten ears of rabbits show that total blood flow is for a time greater than that in normal ears and does not become completely arrested until more than 50 hours after injury (4, 6). During the intervals characterized by rapid blood flow, tests with fluorescein show both delayed entrance and delayed exit of dye from the skin of the injured region. Lange's recently reported observations in which similar fluorescein tests were used upon accidental and experimental cold injury also point out the paradox of impaired dye exchange in the presence of good blood flow (1). He observed diminished fluorescence in regions injured by mild cold and was able to show that capillary blood obtained from poorly staining regions contained as much fluorescein as that from normal, brightly fluores-

cent skin. Two possible explanations were offered to account for the differences observed: namely, that capillary permeability had decreased as the result of cold injury and that local vasoconstriction resulted in reduction of filtration pressure. The second of these explanations was considered to be a local change probably not involving more than axon reflexes, since the difference could not be abolished by local anesthesia or by sympathetic block. Severe cold, sufficient to freeze the tissues, resulted in delayed entrance of fluorescein and apparent hyperfluorescence when observations were made at successively longer intervals after dye injection.

The present studies on gangrene resulting from cold injury include several observations which are difficult to reconcile with Lange's assumption that reduced capillary permeability was the factor responsible for the abnormal distribution of fluorescein in tissues injured by cold. The rapid swelling of frostbitten parts, which begins virtually with the return of blood flow after thawing, and the high protein concentration of the edema fluid are changes not consistent with decreased capillary permeability. The delayed penetration of fluorescein into frostbitten regions also seems difficult to explain upon the basis of Lange's second suggestion, decreased filtration pressure, since this would have to coincide with the appearance of massive edema and the rise of subcutaneous tissue pressure to levels as high as 25 cm. of water (7).

An attempt to explain the abnormal distribution of dye upon the basis of simple changes in capillary permeability would require the additional assumption that the permeability changes be selective with respect to the plasma constituents as well as to the direction of their free passage. If decreased capillary permeability were responsible for the delayed entrance of dye into the skin of the frostbitten region, the capillaries would have to be capable of passing large quantities of plasma protein into the interstitial fluid during the time that the loss of fluorescein from the blood in the same region was being restrained. Data presented in a previous report (7) furnish ample evidence of protein loss from the blood into the frostbitten ears and feet of rabbits.

Comparison between the fluorescence of frostbitten regions and the adjacent, uninjured areas at about 1 hour after the injection of dye shows that the glow from the injured region is much brighter than that from the normal area. This is an example of the "hyperfluorescence" of skin subjected to severe cold injury, described by Lange and Boyd (1). However, serial measurements made from the time of injection of dye furnish evidence on two important points: (a) the maximum brightness of the two areas does not coincide in time; and (b) the intensity of fluorescence at maximum for injured areas is lower than the maximum intensity reached in normal skin. Thus, what, on first inspection, might seem to be a hyperfluorescence of the injured area is actually less than the maximum intensity reached at an earlier time in normal skin. The appearance of hyperfluorescence is the result of removal of dye from adjacent uninjured areas at a faster rate than from the frostbitten area. This circumstance again poses the problem of apparently selective changes in permeability. If it be assumed that the hyperfluorescence is an expression of increased capillary permeability (1), then it must be a selective form of change in permeability, manifesting a decreased restraint of dye as it leaves the blood stream in the injured region and then failure of fluorescein to move into the capillaries when the diffusion gradient has become reversed by the decline of concentration of dye in the blood. Some form of permeability change, which is peculiarly selective with respect to particle size and which operates only in one direction, must be postulated to account for the facts. Such a postulate departs widely from the accumulated experience of investigators who have studied the nature of exchanges across capillary membranes (8, 9, 10).

Both theoretical considerations (10) and measurements on normal animals (2) indicate that fluorescein should become distributed in a manner similar to that observed for other relatively small molecules, *i.e.,* largely by diffusion. The major factors involved in such transfers of dissolved material include the diffusion gradient, the permeability of capillaries, the blood flow rate, and the surface area available for diffusion. The evidence presented above shows that the dye is slow in entering and still slower in leaving areas injured by severe cold. At the same time, other evidence indicates that the diffusion gradients, the minute

volume blood flow, and the degree of capillary permeability are all such that faster rather than slower transfer of dye would be expected. As to possible alteration of the surface area available for diffusion, direct observation of blood flow in frostbitten, transilluminated ears of rabbits has shown that stasis begins early in the true capillaries and is complete within 10 minutes of the return of blood flow after thawing. Flow persists in arteriovenous anastomoses and arteriolarvenular capillaries. Since protein loss into the edema fluid is high in the early period and lower at later stages, it is not difficult to conceive of the early loss as occurring via abnormal leakage from these capillaries, which later are prevented from participating as exchange surfaces by having been blocked by silting red blood cells. Thus, blood flow persists at large minute volume and relatively high pressure in the channels remaining patent. This circumstance serves to explain both the rapid production of edema fluid by filtration and the interference with fluorescein distribution; there is a sharp reduction in the area available for diffusion. The dye, which is filtered out from the arteriorlar-venular capillaries, finds its way back into the blood stream, not by the usual reversal of gradient as the concentration of dye in the blood declines, but by slower removal via lymphatics and by movement of dye into adjacent uninjured regions where stasis has not occurred.

The above interpretation of phenomena observed in the aplication of fluorescein tests to the study of frostbite is consistent with other manifestations of altered blood flow and fluid exchange occurring after severe cold injury. Information about the degree of involvement of capillaries by stasis and its resolution may provide helpful guides in treatment and prognosis. Thus, in addition to the use of fluorescence as a clear-cut indicator of the presence or absence of blood flow, the estimation of rate of dye exchange in injured tissues may be of clinical value.

SUMMARY

Analyses were made of the distribution of intravenously injected fluorescein in normal ears of rabbits and in ears severely injured by cold. The results showed that both the dye's entrance into and its exit from the injured regions were slower than the exchanges observed in uninjured tissues. The maximum intensity of fluorescence reached in frostbitten skin was lower than the maximum recorded in normal skin. Repeated tests on the same animal showed that the exchanges of dye became progressively slower with time after injury.

When both ears of rabbits were frostbitten and procaine block of the stellate ganglion was produced on one side, the tests with fluorescein showed earlier and higher maximum intensity of fluorescence in the frostbitten ear on the side blocked. This maneuver had been shown in other experiments to be followed by delay in the onset of stasis. The greater intensity of fluorescence on the side having increased blood flow as the result of blockage of the stellate ganglion was attributed to the longer persistence of blood flow through true capillaries and, hence, greater opportunity for exchange of dye between the blood and interstitial fluid.

Edema fluid was taken from frostbitten feet of rabbits at a sufficiently long interval after the intravenous injection of fluorescein to permit the concentration of dye in the blood to fall to very low levels. Analysis of the edema fluid showed that the fluorescein concentration was at least twice as great as that in the blood.

The mechanisms of impaired dye exchange in frostbitten tissues are discussed, and a hypothesis is presented in which the impairment of dye exchange is attributed to reduction of available surface for diffusion by the development of stasis in the true capillaries following cold injury.

BIBLIOGRAPHY

1. Lange, K., and Boyd, L. J., The functional pathology of experimental frostbite and the prevention of subsequent gangrene. Surg., Gynec. & Obstet., 1945, 80, 346.
2. Crismon, J. M., and Fuhrman, F. A., Studies on gangrene following cold injury. IV. The use of fluorescein as an indicator of local blood flow: distribution of fluorescein in body fluids after intravenous injection. J. Clin. Invest., 1947, 26, 259.
3. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. I. A method for producing gangrene by means of controlled injury by cold. J. Clin. Invest., 1947, 26, 229.
4. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. II. General course of events in rabbit feet and ears following untreated cold injury. J. Clin. Invest., 1947, 26, 236.

5. Fisher, R. A., Statistical Methods for Research Workers. Ed. 6, Oliver and Boyd, London, 1936.
6. Crismon, J. M., and Fuhrman, F. A., Studies on gangrene following cold injury. VI. Capillary blood flow after cold injury. J. Clin. Invest., 1947, **26**. (In press.)
7. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. III. Edema following cold injury: its magnitude and the composition and source of edema fluid. J. Clin. Invest., 1947, **26**, 245.
8. Zweifach, B. W., The structural basis of permeability and other functions of blood capillaries. Cold Spring Harbor Symp. Quant. Biol., 1940, **8**, 216.
9. Danielli, J. R., and Stock, A., The structure and permeability of blood capillaries. Biol. Rev., 1944, **19**, 81.
10. Teorell, T., Kinetics of distribution of substances administered to the body. II. The intravascular modes of administration. Arch. Internat. de Pharmacodyn. et de Thérap., 1937, **57**, 226.

EFFECT OF CREATINE ON THE PAIN OF MUSCLE ISCHEMIA

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The stimulus of the pain which arises from exercising ischemic muscle has been ascribed to a "factor P" (1). It has been suggested that this factor may be lactic acid, a compound of phosphorus, or creatine, or ammonia (2); phosphoric acid (3); potassium (4); lactate ion (5); or histamine (6). On the other hand, conclusions have been reached that potassium, sarcolactate, and ammonium ions are probably not responsible (6).

No investigations of the relation of creatine to the pain of muscle ischemia have been reported although such a relationship has been suggested (2). In the present study, the pain in exercising ischemic muscle was studied before and during creatine administration in order to observe the effect of creatine upon the pain process.

METHODS

Healthy male medical students were used as subjects with 6 in the control group and 8 in the experimental group. Each subject ate the usual diet of his choice and collected his total 24-hour output of urine. At 3:00 P.M. each day the subject brought the urine to the laboratory and submitted to a standard procedure (7) for the determination of the duration of exercise before the appearance of "definite" pain in the exercising ischemic muscle. The endpoint chosen was the first appearance of a definite sensation of pain in the forearm or hand after exercise had been executed at the rate of one contraction per second. The time, in seconds, was recorded by a stop watch concealed from the subject. The right arm alone was used in all experiments. Instead of simple flexion of the fingers to form a fist, the subject squeezed a gripping device against a spring tension of 1.1 kgm.

The urinary creatine and creatinine were determined in duplicate by the method of Peters (8), using the Evelyn photoelectric colorimeter for the color comparisons. Urine studies were made during both the preliminary control and the experimental periods.

Following a period of several days during which the subject became familiar with the procedure, single observations were made daily for a control period of 2 days before creatine administration was begun.

Creatine, C.P., was dissolved in water, chilled, flavored with citric acid and saccharin, and administered in daily doses of 10 grams for a period of 4 days. Creatine was given only after 2 days of preliminary studies. The control subjects were given flavored water, from a flask labelled "creatine," and were led to think that they were being given creatine. No one was informed of the grouping of subjects or of the exact nature of the experiments.

RESULTS

The exercise duration, in seconds, until the appearance of a definite sensation of pain in the exercising ischemic muscles was recorded during the 2-day preliminary period. For each subject the mean of these two values is plotted in Figures 1 and 2 on the zero line of the control period. Each of the two values is then plotted in terms of per cent variation from the mean value. During the experimental periods, the time of appearance of pain is plotted as per cent variation from the mean value of the preliminary control period.

Control subjects. In each of the 6 subjects, the time of appearance of definite pain in the preliminary control period did not vary more than 5.5 per cent from the mean of the two values (Figure 1).

On the first day after receiving the flavored water, to be designated hereafter as "placebo," only 2 subjects (C. H.; R. M.) observed definite pain within the time range of the preliminary control period. One of these, R. M., again observed pain within the control range on the second day. The only definite trend toward uniformity of response was the relatively delayed pain appearance in five of the subjects on the fourth day of the experimental period, as shown in Figure 1.

Creatinuria was not observed in any of the control subjects at any time.

Subjects receiving creatine. On the first day of the experimental period, 5 of the 8 subjects observed definite pain either within the limits of the preliminary control period or earlier. By the fourth day, when creatine retention was at a maximum, 6 subjects observed pain later than it

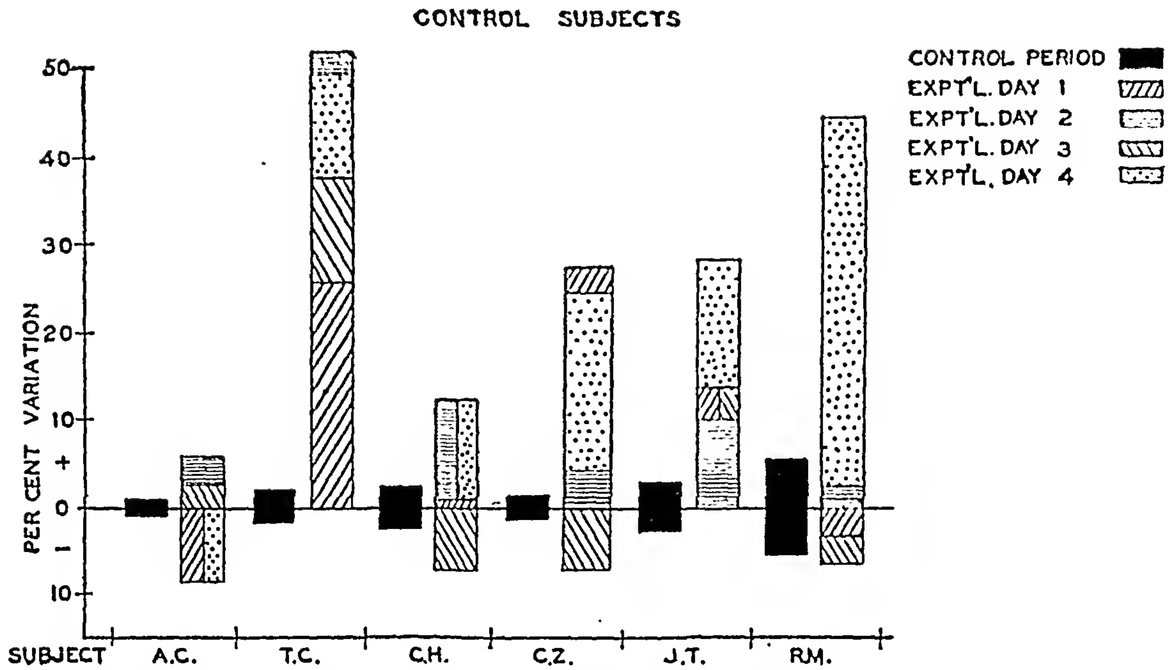

FIG. 1. PER CENT VARIATION IN TIME OF APPEARANCE OF DEFINITE PAIN IN EXERCISING ISCHEMIC MUSCLE DURING PRELIMINARY CONTROL PERIOD AND DURING EXPERIMENTAL PERIOD WHEN PLACEBOS WERE GIVEN

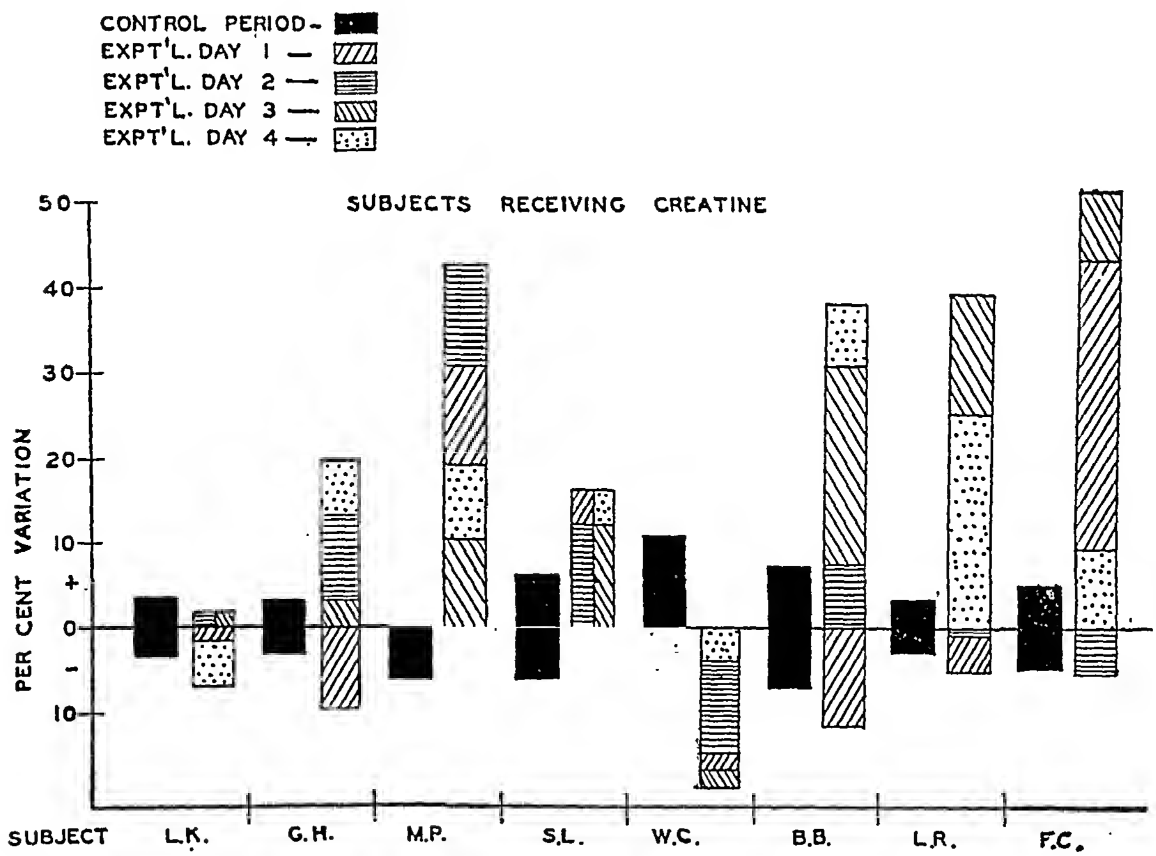

FIG. 2. PER CENT VARIATION IN TIME OF APPEARANCE OF DEFINITE PAIN IN EXERCISING ISCHEMIC MUSCLE DURING PRELIMINARY CONTROL PERIOD AND DURING EXPERIMENTAL PERIOD WHEN CREATINE WAS GIVEN

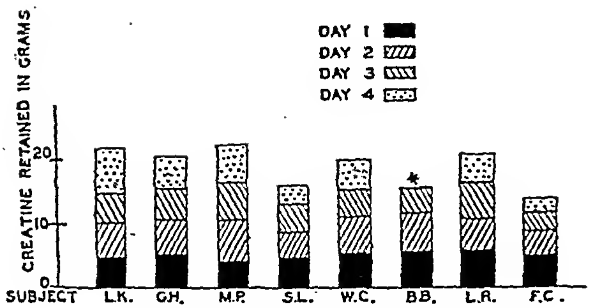

FIG. 3. CREATINE RETENTION, IN GRAMS, DURING EXPERIMENTAL PERIOD WHEN CREATINE WAS ADMINISTERED

* Urine specimen lost.

had appeared during the control period, as shown in Figure 2.

Creatine retention. The subjects were on their usual diets, which were not creatine-free; so an arbitrary method of calculation was adopted. The creatine retained was considered to be the difference between that which had been fed and that excreted through each successive day of the experimental period. This value is recorded as the retained creatine and is plotted in Figure 3. The maximum retention was 22.60 grams of creatine in subject M. P., and the lowest was 13.79 grams in subject F. C.

DISCUSSION

It has been reported (9, 10) that the creatine concentration of muscle is increased by the feeding of creatine. In our subjects, there was a definite trend toward delayed appearance of pain in exercising ischemic muscle as the quantity of retained creatine increased. Perhaps, then the pain-producing phenomenon in ischemia is related to the creatine content of the muscle. However, a similar delay in pain appearance occurred in the control subjects. Since the two groups reacted in a similar manner and since the controls were not receiving creatine, it appears definite that increased creatine storage is not the fundamental cause of the delayed pain response. Training, or possibly some psychological factor, may be involved but this is mere speculation.

In comparing the amount of exercise required to produce pain in the two arms of 7 subjects, Katz, *et al* (11) found that pain appeared earlier in the left arm. The difference in the two arms was ascribed to training, as the right hand had been used much more frequently in the clenching exercise. We have used the right arm alone in all experiments and have made a single determination daily for not longer than 8 days. It would seem that training, under these conditions, would play not more than a minor role. In addition, several subjects showed marked shortening of the period of exercise on the first, second, or third days of the experimental period. If training is the major factor in producing the general tendency toward the longer toleration of exercise, it certainly does not influence the results in a graded uniform fashion.

SUMMARY

There was a definite trend toward delayed appearance of pain, in a majority of 8 subjects, as the quantity of retained creatine increased. However, the same tendency was observed in the control group which was receiving a placebo only. It is concluded that increased concentration of muscle creatine is not the primary cause of the delayed appearance of pain. Training may be involved in the delay but some unexplained factor, or factors, seems more likely.

BIBLIOGRAPHY

1. Lewis, T., Pickering, G. W., and Rothschild, P., Observations upon muscular pain in intermittent claudication. Heart, 1931, 15, 359.
2. Kissin, M., The production of pain in exercising skeletal muscle during induced anoxemia. J. Clin. Invest., 1934, 13, 37.
3. Katz, L. N., Mechanism of pain production in angina pectoris. Am. Heart J., 1935, 10, 322.
4. Harpuder, K., and Stein, I. D., Studies on the nature of pain arising from an ischemic limb. II. Biochemical studies. Am. Heart J., 1943, 25, 438.
5. Elliot, A. H., and Evans, R. D., Ischemic pain in exercising muscles. Am. Heart J., 1936, 12, 674.
6. Maison, G. L., Studies on the genesis of ischemic pain: The influence of the potassium, lactate and ammonium ions. Am. J. Physiol., 1939, 127, 315.
7. Harrison, I. B., and Bigelow, N. H., Quantitative studies of visceral pain produced by the contraction of ischemic muscle. A. Research Nerv. & Ment. Dis., 1943, 23, 154.

8. Peters, J. H., The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., 1942, 146, 179.
9. Chanutin, A., and Silvette, H., The influence of fasting and creatine feeding upon the creatine content of the tissues and blood of the white rat. J. Biol. Chem., 1928, 80, 589.
10. Chanutin, A., and Beard, H. H., A study on the effect of feeding creatine on growth and its distribution in the liver and muscle of normal mice. J. Biol. Chem., 1928, 78, 167.
11. Katz, L. N., Lindner, E., and Landt, H., On the nature of the substance (s) producing pain in contracting skeletal muscle: Its bearing on the problems of angina pectoris and intermittent claudication. J. Clin. Invest., 1935, 14, 807.

SCLEROMA: AN ETIOLOGICAL STUDY

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Rhinoscleroma was discovered in 1870 by von Hebra (1) who considered it an unusual form of skin cancer. Geber (2) later characterized the lesion as a chronic inflammatory process accompanied by perivascular cellular infiltration and connective tissue proliferation. Mikulicz (3) clearly described the histological picture including the diagnostic "foam" or "lace" cells which have been named after him.

Since the disease is not confined to the nose as von Hebra originally assumed in using the prefix "rhino," the more inclusive term scleroma was officially adopted at the Second International Congress of Otorhino-laryngology (4) held in Madrid in 1932.

PATHOLOGY

Scleroma is a disease which clinically is well defined. It is a granulomatous lesion primarily localized in the upper respiratory tract. Secondarily it may appear about the pyriform aperture of the vestibule of the nose. In the respiratory tract, scleroma is most often localized in the anterior portion of the nose, in the vestibule or the posterior nares. In the larynx, the lesions are found usually in the subglottal space.

The pharynx and throat are the seat of lesions in a third of the cases. These are found in the mesopharynx, the posterior surface of the soft palate, the uvula, the pillars, and the lateral portion of the posterior wall. The trachea is occasionally involved.

The gross lesion most frequently described is the circumscribed nodular type which may appear as a single nodule or in groups. Each nodule may appear as a single nodule or in groups. Each nodule may vary in size from 1 to 10 mm. Their consistency varies; the early infiltrates are soft while those of longer standing are harder.

Histologically, the picture is that of a granuloma. The ciliated epithelium is transformed into stratified pavement cells. In the deeper layers of the cornea, the protoplasm becomes more dispersed and partially disappears; vacoules and pyknotic nuclei are seen. Perivascular infiltration is found in which the Mikulicz cell stands out.

Clinically, the nodular type is easily recognized. However, one may assume the existence of a prenodular stage which is less likely to be recognized since there have been no *accepted* specific tests for the disease. Szmurlo (5) has actually described this form of scleroma as the "diffuse" stage. This first "appears as atrophic rhinitis characterized by atrophy of the nasal mucosa with abundant secretion and gray crusts on the surface."

The clinical course of the disease is benign, the onset is insidious, and the duration may be from twenty to thirty years. There is little effect on the general health except for the mechanical obstruction of the air passages which may in extreme cases lead to asphyxia.

INCIDENCE

Scleroma was once thought to be confined to Central and Southeastern Europe, but it is now recognized more often and is generally acknowledged as world-wide in occurrence. In a comprehensive report, Cunning and Guerry (6) in 1942 reported that 102 cases had been described to that date in the American and Canadian literature. Streit (7) contends that an endemic area exists in Central Europe especially Poland. Lasagna (8) feels that it is becoming more common in Italy, and there is some evidence that the disease is seen frequently in Central and South America.

ETIOLOGY

The problems of scleroma are clouded by the confusion associated with its etiology. In 1882, von Frisch (9) described an organism which he asserted to be the cause of the disease. Due to inadequate morphological studies inherent in the embryonic state of the science of bacteriology and due to the lack of an experimental lesion in

animals resembling the disease in human beings, Frisch's organism was not universally accepted. The belief expressed by the American, Perkins (10), that the scleroma bacillus was not related to the disease, has been echoed periodically since then, and the current opinion may be seen in the comments of two outstanding bacteriological texts. Gay (11) states, "*K. rhinoscleromatis* bears some as yet obscure relationship with the disease rhinoscleroma. It appears, however, commonly to be a secondary invader in the lesions characteristic of the disease." Wilson and Miles (12) state, "There is very little evidence to show that this organism is primarily responsible for it. There is no means by which it can be distinguished with certainty from other members of the capsulated group; and since we know that members of this group may be present in the nose of healthy persons, it is difficult to prove that they play any part in production of rhinoscleroma. The probability is that they are mere secondary invaders, which grow freely in the nose of patients suffering from the disease."

Much of the etiological difficulty encountered is due to the alleged presence of the scleroma bacillus or related organisms in normal throats. To test this hypothesis, we must establish a criterion for the characteristics of the scleroma bacillus, then we must study its occurrence in the normal throat. The very early literature must be discarded since the required facts about the related Friedlander bacillus and the coli-aerobacter group were not available to these first investigators. There is, however, a remarkable uniformity which has been overlooked in the description of the rhinoscleroma bacillus by a number of reliable investigators in recent years. Figi and Thompson (13) in 1928, described 3 strains isolated from patients at the Mayo Clinic. Morris and Julianelle (14), apparently unaware of Thompson's report, studied 9 strains from cases in Sumatra and this country. Both of these workers found their strains to ferment glucose, maltose, and mannite with the production of acid only. Sucrose was fermented slowly by some strains, lactose never. Indole was not formed, nitrate was reduced. Other reactions are noted but are not essential to the differential identification of the organism. Gonsiorowski and Meisel (15), two

TABLE I

Cultural characteristics of scleroma strains

Strain	Mannite	Maltose	Dextrose	Lactose	Sucrose	Nitrate	Indole
1L	A	A	A	–	A	+	–
2F	A	A	A	–	–	+	–
3J	A	A	A	–	–	+	–
4M	A	A	A	–	–	+	–
5R	A	A	A	–	–	+	–
6C	A	A	A	–	–	+	–
7CA	A	A	A	–	–	+	–
8T	A	A	A	–	–	+	–

Polish workers with a large number of freshly isolated strains at their disposal, reported the fermentation of glucose (acid only) but not of lactose, the only sugars tested.

We have isolated seven strains from cases to be described later in this paper and have obtained one culture from the American Type Culture Collection. The results of cultured studies on these organisms are given in Table I.

From the above it is evident that there is a remarkable uniformity in the reactions of our organisms identical with those obtained by the workers mentioned above. We feel that the characteristics described in Table I, including the variable, slow fermentation of sucrose should be considered specific for these organisms. If we include the colony characteristics (large translucent, mucoid, coalescing colonies on blood agar, nutrient agar, and E. M. B. medium), we have a sufficient differential description to identify this organism.

No other related gram negative rod found on the human being has been reported which gives the same pattern of reactions. This is true even of the variable Friedlander group. Julianelle (14), who has done most of the work on the classification of this genus, found no strain in any of the groups A, B, C or the unclassified Friedlander group to give reactions similar to those described for the rhinoscleroma bacillus. We have studied strains of the Friedlander bacillus obtained from the American Type Culture Collection, and find no resemblance of any of these to the scleroma organism (Table II).

From the above and from Julianelle's findings, we concluded that it should be possible to separate the mucoid Friedlander from the mucoid scleroma bacillus.

TABLE II

Cultural characteristics of Friedlander strains

Strain	Mannite	Maltose	Dextrose	Lactose	Sucrose	Nitrate	Indole
132	AG	AG	AG	AG	AG	+	−
8044 A	AG	AG	AG	AG	AG	+	−
4727	AG	AG	AG	AG	AG	+	−
8045 A	AG	AG	AG	AG	AG	+	−
8046 A	AG	AG	AG	AG	AG	+	−
6540	AG	AG	AG	AG	AG	+	−
7380 B	A	A	A	A	A	+	−
9997	A	A	A	A	A	+	−
4208	A	A	A	A	A	+	−

We next tested the assumption that the scleroma bacillus is found in normal throats. A total of 204 normal individuals and 63 with various nose and throat ailments were cultured. The procedure used was found to the satisfactory in the isolation of the rhinoscleroma organisms in the known infected cases. Nose and throat swabs were streaked on blood agar and E. M. B. in all infected individuals. Since the results on the blood agar and E. M. B. were similar, we employed only the latter in our studies of the normal, and the nose and throat cases.

Gram negative organisms (which morphologically are related to the scleroma bacillus) in the nose and throat that will grow on E. M. B. include members of the coli-aerogenes and Friedlander groups, Proteus, Pseudomonas and Alkaligenes. Most noses and throats did not show a sufficient number of any of these to give a positive culture. Of a total of 267 individuals tested, only 34 gave positive cultures; the results of cultural studies of these organisms are presented in Table III.

TABLE III

Cultural characteristics of gram negative bacilli isolated from normal individuals or nose and throat cases

Mannite	Maltose	Dextrose	Lactose	Sucrose	Nitrate	Indole	Number of strains
AG	AG	AG	AG	AG	+	−	13
AG	AG	AG	AG	AG	+	+	2
AG	AG	AG	AG	AG	−	−	1
AG	AG	AG	—	AG	+	+	1
AG	AG	AG	AG	−	+	+	2
AG	A	A	AG	AG	+	−	1
AG	AG	AG	A	AG	+	−	1
AG	AG	AG	A	−	+	+	1
AG	AG	AG	−	−	+	−	2
AG	AG	AG	−	−	+	+	1
A	A	A	A	A	+	−	1
−	−	A	−	−	−	−	1
−	−	AG	−	−	+	+	1
−	−	A	−	−	+	+	1
−	−	−	−	−	−	−	4
−	−	A	−	−	+	−	1

Although we have not as yet speciated the above strains, it is obvious that none has the pattern we have found to be characteristic of the scleroma bacillus. Further work is in progress to name these organisms as to species.

SEROLOGICAL STUDIES

In order further to relate the organism to the disease in question, serological studies should be of value. There are many references in the literature to such studies. As is true of the cultural studies, the early serological reports are confusing, and for the same reasons mentioned previously. However, more recent studies, if correlated, certainly are not to be disregarded. Goldzieher and Neuber (16), Brault and Masselot (17), and Quast (18) have all agreed that serum from patients with scleroma or from rabbits immunized with the organism is capable of fixing complement. Fitzgerald (19) showed that a rabbit immune serum agglutinated specifically the decapsulated organism. Prasek and Prica (20) and Morris and Julianelle (14) have both demonstrated the specific antigenicity of the organism.

We have employed the complement fixation test in our studies. Seven patients suffering from scleroma have given positive reactions with antigen consisting of heat-killed (65° C. for 30 minutes) organisms grown on nutrient agar slants.

Over 500 sera from normal patients and from individuals with an assortment of other diseases have been tested with only occasional positive reactions. However, we find such "false positive reactions" to be a function of the concentration of antigen and antiserum. We are now in the process of standardizing the procedure to obtain maximum accuracy. Details of the test will be published elsewhere.

CASE STUDIES

One further controversial question concerning scleroma has been its contagiousness. Its endemic nature has been suggested previously. Familial occurrence would indicate an infectious etiology. Lasagna (8) Robertson and Secretan (21), and Gerber (22) have reported duplicate cases occurring in the same family. We have studied seven cases, six of which have occurred in the same family.

Case 1. L. M., male, age 20, of Mexican descent, was referred to the clinic because of dyspnea and a cough productive of a thick yellow mucoid material.

About one year ago he began to notice that his nostrils were "plugged up" and that a sticky yellow discharge was chronically present. The significant physical findings related to the respiratory tract. A hard granulomatous mass filled the nares, nasopharynx, and the pharynx. In some places the mass was covered by dirty, green, malodorous crusted material which was occasionally expectorated. The voice was hoarse and there was some granulation involving the epiglottis and aryepiglottic folds. The patient complained that the dyspnea interfered with normal activity. There were no other significant laboratory or physical findings.

Biopsy of the lesion demonstrated a typical granuloma with plasma and lymphoid cells. Hyalinization was present. Mikulicz cells were numerous and easily demonstrated. Staining with the Krajian modification of the Gram-Weigert method showed numerous bacilli present, extra- and intracellularly.

The patient has been treated with sulfadiazine, penicillin, and streptomycin. Only the last was really effective against the organism *in vitro* and caused a decrease in the number of rhinoscleroma bacilli in the throat, but did not affect the lesions after 10 days of treatment with 10 grams of the drug. Deep x-ray is being used at the present time.

From the above patient we isolated the scleroma bacillus in twenty consecutive throat cultures on blood agar and E. M. B. agar. The organism grows as a large, mucoid, flowing colony easily picked from the usual throat contaminants after 24 hours of incubation.

On questioning this first patient, we found that his father has similar symptoms, of longer duration. His history follows:

Case 2. L. M., age 37, was essentially well until his nose was broken about 10 years ago. After this his nose became progressively "stuffy" and a thick yellow discharge developed. Rhinoscleroma was diagnosed clinically elsewhere one year after onset and x-ray therapy was given. At present, there is adequate airway, but the lining of the nose and pharynx is hard, irregular, and studded with foul smelling, greenish crusts.

Biopsy findings were typical and identical with those of Case 1, including the presence of the small bacilli in stained sections of the tissue. Organisms identical with the scleroma bacillus have been isolated repeatedly in cultures from the nose and throat.

After seeing that the father and son both were suffering from the same disease, we thought that other members of the family might show symptoms of a similar nature. From our discussion with father and son, we knew that they were suffering from vague symptoms associated with the upper respiratory tract. In addition to the son (Case 1) the family consisted of four daughters, a young son age 9, and the wife of the oldest son (Case 1). The mother of the children has been separated from the family for the past four years and is not available.

The wife of Case 1 and the youngest son are free of symptoms and culturally negative. The four girls seem to show various stages of an infection of the nasopharynx which resembles the "diffuse" type of scleroma described by Szmurlo, and mentioned previously. Their case histories follow:

Case 3. J. M., age 19 (sister of Case 1), is asymptomatic except for frequent "head colds" and a watery nasal discharge which has been chronically present for two years. There is marked atrophy of the inferior and middle turbinates and the nose contains crusts and casts of greenish-yellow matter that has the same foul odor noticed in others of her family.

Biopsy sections show inflammation and the presence of many monocytes. The mucous glands are hyperplastic. The sections stained for bacterial examination were inadequate in this case. Cultures revealed the presence of the scleroma bacillus in the throat and nose.

Case 4. C. M., age 17 (sister of Case 1), is asymptomatic except for a chronic cough which she attributes to a thick, sticky, nasal and postnasal discharge. She had "lobar pneumonia with empyema" three years ago. Recovery followed open drainage. Physical examination of the upper respiratory tract showed marked atrophy of the inferior and middle turbinates with some thickening of the septum superiorly and anteriorly, and the presence of greenish crusts.

Biopsy sections show a diffuse infiltration, granulomatous in type, under the epithelium. Occasional cells of the macrophage variety are present. There are present also large foamy cells not typical of the Mikulicz cell. Bacterial stains show bacilli to be present extracellularly. Repeated cultures of the nose and throat show the presence of the scleroma bacillus.

Case 5. M. M., age 14 (sister of Case 1), complains of increased nasal "stuffiness" of a year and a half duration, with no visible discharge. Physical examination shows almost complete obstruction due to the involvement of the anterior and of the inferior turbinates. The hypertrophied tissue causing obstruction has the appearance of granulation tissue and bleeds easily on touching. There was some crusting due to greenish-yellow purulent exudate.

Biopsy sections show tissue consisting of large strands of hyaline material, and of compact masses of small round cells, mostly the macrophage type. In some areas the tissue appears necrotic with the cells staining faintly. One area shows mucous vacuoles which resemble fat tissue; these are surrounded by round cells of macrophage type. Some of the cells show foamy cytoplasm and some

are binucleate. No typical Mikulicz cells are seen. No bacterial stain was done. Repeated cultures of the nose and throat showed the presence of the scleroma bacillus.

Case 6. R. M., age 11 (sister of Case 1), is asymptomatic although her sisters complain that she is always clearing her throat. Examination showed considerable hypertrophy of the tonsils. No atrophy, granulation or crusting was present in the nares.

Biopsy of a slightly injected area of the pharyngeal mucosa reveals the presence of stratified and ciliated epithelium below which there are occasional areas of infiltration of macrophages. Small granulomatous lesions are found here and there around capillary vessels. Noticeable is the presence of large cells with peripheral nuclei and foamy cytoplasm. The bacterial stains show bacilli inside the macrophages. The scleroma bacillus was repeatedly isolated from nose and throat cultures.

The last four cases, in our opinion, resemble varying stages of rhinoscleroma. In none of these cases were the symptoms sufficiently severe to result in the patient being referred to a doctor, yet physical examination reveals lesions which at least in three of the cases are suggestive of the disease. In one case, R. M., the youngest sister, there are practically no gross pathologic lesions and yet the microscopic picture in all the cases including the last confirms the existence of the disease syndrome, which, together with the isolation of the scleroma bacillus in each case, gives extremely presumptive evidence that here we have a family in which six members are afflicted with various stages of the disease rhinoscleroma. Serological findings in each of these cases gives further evidence for this contention, although until the complement fixation test, mentioned previously, is standardized, emphasis on the serology must be avoided.

One further case will be presented although it is unrelated to the family mentioned above. This case also gave a positive culture for the scleroma bacillus and a positive complement fixation test.

Case 7. L. C., age 63, female of Mexican descent. The patient was seen first fourteen years ago when she complained of obstruction to her breathing of four months' duration. At that time, "granular pharynx" and hypertrophy of the right mid-turbinate accompanied by postnasal purulent discharge were reported. The patient was not seen again until recently at which time in addition to the previously mentioned symptoms the inferior turbinate on the right also was reported to be hypertrophic and irregular with the appearance of fungating granulation tissue.

Biopsy sections show the presence of shreds of epithelium, dense fibrous strands, and compact masses of round cells. Under high power the cells appear to be mostly plasma cells and lymphocytes, but here and there are seen numerous macrophages with foamy cytoplasm. An occasional large cell with peripheral nucleus and pink cytoplasm is seen. No hyaline globi are visible. Lesions seem to be of the granulomatous type.

CONCLUSION

Six cases of scleroma in the same family are described showing varying stages of the disease. The etiologic agent is suggested to be the scleroma bacillus first described by Frisch. Its characteristics are given. The incidence of this organism in the normal population has been studied. Suggestive serological findings are reported.

BIBLIOGRAPHY

1. von Hebra, R., Ueber ein eigenthumliches Neugebilde an der Nose, Rhinosclerom, nebst histologischem Befunde vom Dr. M. Kohn. Wien. med. Wchnschr., 1870, 20, 1.
2. Geber, E., Ueber das Wesen des Rhinosclerom; eine klinischhistologische Studie. Arch. f. Dermat. u. Syph., 1872, 4, 419.
3. Mikulicz, Johann, Ueber das Rhinosclerom. Arch. f. klin. Chir., 1877, 20, 485.
4. Rapp, Cong. Internat. d'oto-rhinolaryng., Madrid, 1932.
5. Szmurlo, J., Anatomicopathology of scleroma. Arch. Otolaryng., 1933, 18, 393.
6. Cunning, D. S., Guerry, Du. P., Scleroma, Arch. of Otolaryng., 1942, 36, 662.
7. Streit, H., Etiology of scleroma. Arch. of Otolaryng., 1933, 18, 392.
8. Lasagna, F., Infective scleroma of the respiratory passages. J. Laryng. and Otol., 1925, 40, 369.
9. von Frisch, A., Zur Aetiologie des Rhinoskleroms. Wien med. Wchnschr., 1882, 32, 969.
10. Perkins, R. G., Relation of the bacillus mucosus capsulatus group to rhinoscleroma and of the various members of the group to one another. J. Infect. Dis., 1904, 1, 241.
11. Gay, F. P., Agents of Disease and Host Resistance. Thomas, Springfield, 1935.
12. Wilson, G. S., Miles, A. A., Topley and Wilson's Principles of Bacteriology and Immunity. 3rd Ed., Baltimore, 1946.
13. Figi, F. A., Thompson, L., Rhinoscleroma. J. A. M. A., 1928, 91, 637.
14. Morris, M. C., and Julianelle, L. A., A biological classification of the bacillus of rhinoscleroma. J. Infect. Dis., 1934, 55, 150.
15. Gonsiorowski, N., and Meisel, H., Sur la biologie de bacilles muqueux encapsules. Comptes Rend. Soc. de Biol., 1929, 100, 293.

16. Goldzieher, M., and Nueber, E., Untersuchungen über das Rhinosklerom. Centralbl. f. Bakt. (Abt. I), 1909, **51**, 121.
17. Brault, J., and Masselot, L., Quelques remarques sur le bacille du rhinosclerome (bacille de Frisch). Arch. de med. exper. et d'anat. path., 1910, **22**, 545.
18. Quast, quoted by Figi and Thompson, 1928.
19. Fitzgerald, J. G., A biometrical study of the mucous capsulatus group. J. Inf. Dis., 1914, **15**, 268.
20. Prasek, E., and Prica, M., Agglutinationsreaktion bei Rhinosklerom. Centralbl. f. Bakteriol. (Abt. I), 1928, **108**, 376.
21. Quoted by Mayer, E., Scleroma of the larynx. Am. J. Med. Sci., 1907, **133**, 751.
22. Gerber, P. H., Ueber das Sklerom insbesondere in Ostpreussen. Arch. f. Laryng. u. Rhin., 1900, **10**, 347.

STUDIES ON A VIRUS FROM A PATIENT WITH FORT BRAGG FEVER (PRETIBIAL FEVER)

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During the summer of 1942, an outbreak of a dengue-like illness of unknown etiology occurred at Fort Bragg, North Carolina. The disease, described by Daniels and Grennan (1), was characterized by moderate prostration, fever, splenomegaly, a rash localizing particularly on the anterior aspects of the legs, and a short course. The localization of the rash gave rise to the name "pretibial fever." Epidemiological information indicated that all of the patients in this outbreak were quartered in the same area of the post and that the incubation period was 10 days or longer. Serological tests served to exclude endemic typhus, infectious mononucleosis, brucellosis, and the typhoid-paratyphoid group of diseases. A commission was assigned by the Surgeon General of the United States Army to investigate the epidemiology and etiology of the disease. Working with frozen material in the form of blood, nasal washings, and urine from the patients, the members of the commission concluded: "All tests on this material proved negative as far as isolating or determining the nature of the infectious agent responsible for this disease was concerned" (2).

Similar outbreaks appeared during the summers of 1943 and 1944. The present report describes the recovery of an infectious agent from a patient having Fort Bragg fever in 1944 and summarizes observations on the biological characteristics of this agent. In addition, it presents the results of neutralization tests which indicate that the agent is related to the human infection; and finally, it describes the reproduction of the clinical disease picture in human subjects inoculated with the agent.

PART I

RECOVERY OF A VIRUS AND ITS CHARACTERISTICS

Recovery of the agent from a patient

On August 16, 1944, a 22-year-old white soldier noted a sudden onset of headache, feverishness, and generalized aching. In spite of these symptoms, he continued on duty until August 19 when his symptoms increased following a stimulating injection of tetanus toxoid, and he was hospitalized. Physical examination showed an acutely ill patient with a fever of 102° F., whose only other abnormal physical finding was a questionably palpable spleen. The leukocyte count was 5,750 per cubic mm. An indistinct maculopapular eruption was noted over the pretibial areas on August 21. This rash faded after 2 days. During his illness the patient showed a remittent temperature which reached 103° to 104.5° F. for 5 days. A blood culture taken shortly after the appearance of the rash proved sterile. Two days after defervescence he was completely recovered and was discharged from the hospital. The clinical diagnosis was Fort Bragg Fever.

Two Syrian hamsters and two guinea pigs were injected intraperitoneally with blood freshly drawn from this patient on August 21, 1944, 5 days after onset, when his temperature was 104.5° F. Both hamsters were found dead on the tenth day. No macroscopic abnormalities were found at autopsy and the viscera were bacteriologically sterile. Passage to other hamsters was

[1] Captain, MC, AUS. Part of this work was done by the author while serving as a member of the Commission on Acute Respiratory Diseases and part while Chief of the Communicable Disease Section, Walter Reed General Hospital, Army Medical Center, Washington, D. C.

[2] Work done at Fort Bragg, North Carolina, was supported in part through the Commission on Acute Respiratory Diseases, Army Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, United States Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation, and the International Health Division of the Rockefeller Foundation to the Army Epidemiological Board for the Commission on Acute Respiratory Diseases.

[3] The studies in Cincinnati were supported by the Commission on Neurotropic Virus Diseases, Army Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

not attempted. Both guinea pigs developed fever (105°–106° F.) 8 days after inoculation. On the second day of fever, one of the animals was sacrificed; citrated blood, suspensions of brain, pooled liver and spleen, and tunica vaginalis were obtained and each injected into two guinea pigs by the intraperitoneal route. Cultures of the blood and organs of the guinea pig showed no growth. After a 7-day incubation period, fever developed in guinea pigs of the groups which had received blood and pooled liver and spleen; but no disease appeared in the other animals. A febrile illness was regularly induced in passage animals inoculated with citrated blood from affected guinea pigs. A similar febrile illness was induced and maintained by passage in guinea pigs injected with suspensions of liver and spleen of such animals; after the fourth serial passage, this line was abandoned for convenience. The stock colonies of the guinea pigs and hamsters were free from intercurrent disease at the time, as evidenced by several negative experiments employing intraperitoneal injections of blood from patients with other diseases.

Pathogenicity for animals and eggs

GUINEA PIGS: Guinea pigs developed fever following intraperitoneal injection of citrated blood or intracerebral injection of serum taken from passage animals on their first day of fever. The incubation period was from 4 to 8 days and the febrile course was from 2 to 4 days. Young guinea pigs (200 grams body weight) showed a more prolonged febrile reaction and a shorter incubation period than adult animals. Convalescent animals remained afebrile when reinoculated with infectious passage material. Fever was the only clinical evidence of infection in guinea pigs; none of the several hundred animals used in the 90-odd passages of the agent ever appeared sick. No macroscopic or microscopic pathological changes were consistently noted in guinea pigs sacrificed at various stages of the infection. Inclusion bodies and rickettsiae were searched for in sections and smears of organs stained by Giemsa's method and other techniques; none was found.

RABBITS: The disease induced in rabbits injected with infectious guinea pig material by the intraperitoneal or intracerebral route was essentially identical with that seen in guinea pigs injected with passage material. This mild febrile illness was maintained during four serial passages in rabbits.

HAMSTERS: Adult hamsters which were inoculated intraperitoneally with blood from guinea pigs of the sixth and subsequent passages died after about 8 days. However, the disease was only irregularly reproduced in adult hamsters which were injected with blood from moribund animals of this species. Young hamsters, 40 to 60 grams body weight, proved more susceptible to the experimental infection since the agent was readily maintained by serial intracerebral passage in them. Cerebral tissue from a young hamster used for the fourth transfer titered 10^{-4} when tested in hamsters.

A characteristic, rapidly progressing disease developed in inoculated hamsters following an incubation period of 7 to 16 days. Some hours preceding death, these animals became abnormally irritable and showed slight ataxia. They assumed a hunched position in the darkest corner of the cage, apparently having photophobia associated occasionally with a seropurulent discharge from the eyes. Generalized tremors, but no true convulsions, were occasionally noted. The initial ataxia rapidly progressed to complete paralysis of all four extremities and death usually ensued within a few minutes. Gross and microscopic findings at autopsy were consistently normal except for areas of fresh hemorrhage in the lungs which were frequently observed and were regarded as agonal manifestations. No inclusion bodies or rickettsiae were seen in the histological sections of the central nervous system or viscera.

MICE: Infectious material injected by various routes into mice of different ages and breeds induced no obvious disease. Furthermore, serial "blind" passages were unsuccessful.

EMBRYONATED EGGS: The intravenous injection of 11-day embryonated eggs with infected serum from passage guinea pigs resulted in the death of most embryos after about 7 days of incubation at 96° F. The agent was subsequently maintained for 23 serial transfers in embryonated eggs by the intravenous injection of suspensions of infected embryo liver diluted from 10^{-2} to 10^{-4}. The embryo livers were often dark green in color, but no other abnormalities were noted. No inclusion bodies or rickettsiae were seen in stained smears of embryo viscera, yolk sac, or chorio-allantoic membranes or fluids. Embryonated eggs survived the injection of infected guinea pig plasma into the yolk sac or the chorio-allantoic sac, or onto the chorio-allantoic membrane. However, it was

possible to demonstrate the presence of the agent in yolk sac tissue of embryos which had been injected intravenously with infected egg material and to propagate the agent thereafter by the yolk sac route. Titrations in hamsters of fresh suspensions of infected embryo liver or yolk sac usually yielded end points of 10^{-4}.

Filterability

Plasma from febrile guinea pigs was employed in a few preliminary filtration studies on the agent. For this purpose, the plasma was diluted with an equal quantity of physiological saline solution and passed through Seitz or Corning UF filters. Virus was demonstrated to be present in the filtrate of a Corning fritted glass filter (UF) but not in that from a single Seitz pad. Materials were tested for infectivity by intravenous injection into embryonated eggs, and the presence of the virus in tissues of embryos which died was confirmed by passage to hamsters and guinea pigs. The glass filter used in the experiment did not permit the passage of *Staphylococcus albus* which was added to the infected plasma.

Neutralization tests

Sera from patients convalescent from Fort Bragg fever and sera from recovered guinea pigs were tested for neutralizing antibody against the new agent. Virus for these tests consisted of undiluted guinea pig plasma or dilutions of infected chick embryo liver covering the range of 10^{-1} to 10^{-3}. The technique was as follows: Equal amounts of undiluted serum to be tested and the appropriate dilution of virus were mixed and incubated for 1 hour at room temperature and then injected into groups of two or three adult hamsters. Each animal received 0.5 ml. of the mixture intraperitoneally. The animals were observed for a period of 21 days and deaths recorded.

The results of such neutralization tests carried out on acute and convalescent sera from five cases

TABLE I

Results of neutralization tests in hamsters

Sera		Source and dilution of virus			
		Guinea pig plasma	Chick embryo liver		
Source	Day after onset	Undiluted	10^{-1}	10^{-2}	10^{-3}
Patient A**	5	D10,* D11	D9, D10, D11	D12, S, S	D15, D16, S
	23	S, S	S, S, S	S, S, S	S, S, S
	39	S, S			
Patient B	4	D12, D14	D9, D9, S	D17, D18, S	D13, D13, S
	28	S, S	S, S, S	D20, S, S	S, S, S
Patient C	4	D11, D11			
	15	D15, S			
Patient D	4	D11, D11			
	24	D11, D11			
Patient E	4		D9, D9, D10	D13, D16, D19	D10, D10, D12
	24		D9, D9, D10	D10, S, S	D12, D13, D20
Guinea Pig B99	0		D9, D9, D9		
	24		S, S, S		
Guinea Pig B100	0		D9, D9, D9		
	24		D14, S, S		

* D10—Death on 10th day after inoculation. S—Survived.
** The new virus was isolated from the blood of this patient.

of Fort Bragg fever and two experimental guinea pigs are summarized in Table I. It is evident from the data that the convalescent sera from Patients A and B and from both guinea pigs contained neutralizing substances which protected the hamsters from death.

Storage

Considerable difficulty has been encountered in preserving the agent. Infectivity of blood, whole tissues, or tissues suspended in broth was lost after storage for 24 hours at 20° C., 4° C., or at − 70° C. in sealed glass ampoules. However, viability has been maintained when 20 per cent suspensions of infected embryo tissue were prepared in sterile skimmed milk media, pH 7.2, frozen rapidly in glass sealed ampoules and stored at − 70° C.; in one instance the agent was active after 8 months storage under these conditions. In one experiment, the infectivity of a chick embryo liver suspension diminished after storage at − 70° C. for 24 hours from a titer of 10^{-4} to 10^{-2}; both titrations were made in hamsters. The lability of the virus was further suggested in the tests with human subjects (see Part II). It was found that plasma taken from these patients during the febrile illness and proved to be infectious (actually whole blood was used here) by injection into other human beings, was not infectious for man after storage at − 70° C. for 12 days.[4] Fresh plasma was known to be infectious from work with hamsters.

Relation to other infectious agents

The available data are insufficient to identify this agent with any known pathogen. Sera of guinea pigs convalescent from the experimental disease failed to fix complement with the soluble antigen of lymphocytic choriomeningitis. Recovered guinea pigs were fully susceptible to infection with the Balkan Grippe strain of the rickettsia of Q fever (3). Convalescent sera from three of the patients described in Part II failed to fix complement with the antigen of Rocky Mountain spotted fever; in addition, no significant titers developed in the Weil Felix reaction with OX19, OX2, and OXK. The new agent was clearly different from the rickettsia-like organism which had been previously encountered in guinea pigs used in studies on Fort Bragg fever (4). Subsequent unpublished work indicated that the infection caused by the rickettsia-like agent was enzootic in the guinea pig colony and bore no relation to Fort Bragg fever in human beings.

PART II

PATHOGENICITY OF THE AGENT FOR HUMAN BEINGS [5]

In order to test the disease-producing capacity in man of the virus which had been maintained in guinea pigs and embryonated eggs, the inoculation of human subjects was next undertaken. The plan was to determine whether or not any disease could be produced by the injection of a suspension of infected egg embryo liver and, if infection occurred, to study the resistance to the new agent of individuals known to have recovered from dengue fever and from sandfly fever.

METHODS

Preliminary physical examinations were performed on all subjects; in addition, the results of x-ray examinations of the lungs, routine blood counts, urinalyses, and daily rectal temperatures indicated that they were, with few exceptions, in good general physical condition; all were free of intercurrent infection. Particular attention was given to examination of the skin, where long-standing blemishes might be confused later with the appearance of specific rashes. No attempt was made to isolate the patients because of the lack of epidemiological evidence for cross infection in naturally occurring Fort Bragg fever. It may be mentioned now that subsequent experience in the present studies likewise failed to suggest that the disease is contagious.

The first group of three patients were injected with a 10 per cent suspension of infected embryonated chick liver in saline. The material for this purpose was harvested from living embryos on the sixth day after intravenous injection with the 23rd egg passage, at a time when 50 per cent of the embryos had already died. This egg line had been started from the infectious serum of a guinea pig of the 80th serial passage in this host. Aerobic and anaerobic cultures of the inoculum for patients showed no growth on the usual bacteriological media. This same embryo liver emulsion was infectious at a dilution of 10^{-4} when titrated intracerebrally in hamsters. Mice given a

[4] This might explain the failure in 1942 of the group who investigated Fort Bragg fever to recover the agent from the blood of patients. All of the materials were stored in the frozen state for some days before injection into animals (2).

[5] The human subjects used in this study were part of a group undergoing fever therapy at the Longview State Hospital in Cincinnati.

combined intracerebral and intraperitoneal injection remained well. Guinea pigs which were also injected intraperitoneally with this inoculum developed the characteristic febrile reaction and the agent was readily transmitted to a second group of guinea pigs by inoculation of blood. It is evident that the infectious agent injected into the first group of human subjects had characteristics typical of those previously described for the virus.

The three individuals who made up the first group of subjects received 3.0 ml. intramuscularly and 0.4 ml. intracutaneously of the chick embryo liver suspension described above. Subsequent transfers of the agent to other individuals were made with blood which was drawn from the patients within 24 hours after the onset of fever. This was defibrinated, pooled, and immediately injected into the new subjects who received 5.0 ml. intramuscularly and 0.4 ml. intracutaneously.

RESULTS

A short febrile illness developed in all three of the subjects following injection with the suspension of infected chick embryo liver. The three individuals of the second group, who received pooled defibrinated blood of the first patients, developed a similar short febrile illness. Accordingly, the plan to carry out immunity studies was put into effect in the third group which consisted of two normal individuals, two persons recovered from sandfly fever, and four recovered from dengue fever. All of the patients, with one exception, in this third group developed the febrile reaction, and the majority exhibited the clinical picture of Fort Bragg fever with rash and leukopenia. The single exception was a normal subject who failed to show any fever over a 23-day period of observation. He had not been out of the state of Ohio, as far as could be determined, and his failure of response remains unexplained. The results of these studies in man are summarized in Figures 1 and 2. Figure 3 illustrates the types of rash which were seen. More detailed information concerning the various clinical features of the disease produced in man is given below.[6]

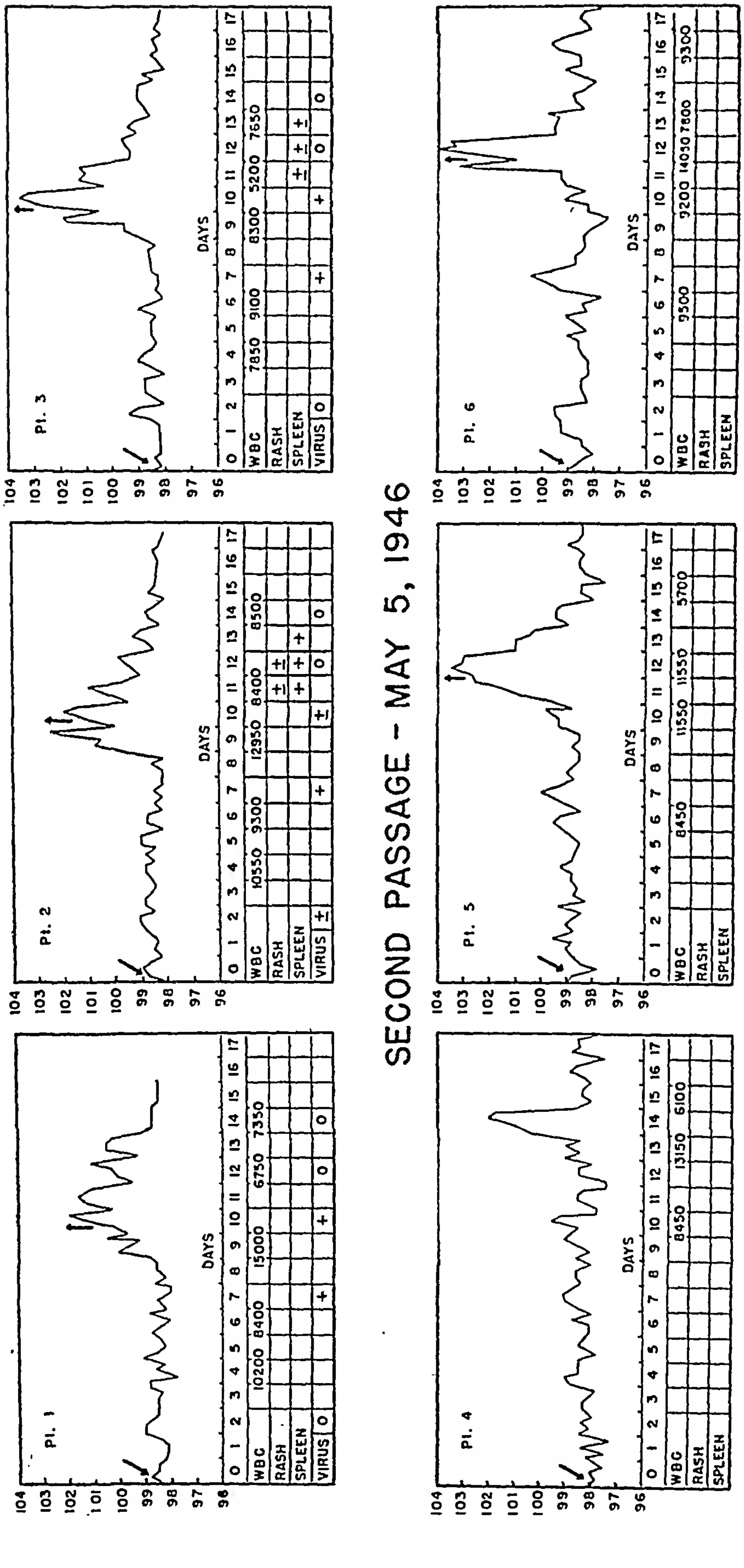
Incubation periods. The three individuals in the first group, inoculated with infected embryo liver suspension, came down sharply with fever on the ninth day after inoculation. The second group developed fever from the 11th to the 14th day after inoculation with defibrinated blood from the first group. The third and largest group, consisting of eight individuals, had incubation periods varying from 8 to 14 days, averaging 11. These were quite in accord with the estimate of 10 days or longer in the naturally occurring disease at Fort Bragg (2).

Fever. The temperature curves of the patients were spiking, such as occurs in the natural disease (1). Daily physical examinations failed to reveal evidence of the occurrence of intercurrent disease. The clearcut temperature elevations, ranging from 101° to 105.5° F. (rectal) and extending over a period of 1 to 6 days, average 3.2, stood out sharply from the preliminary temperature base lines. In four patients, a slight rise in temperature appeared 4 days preceding the main bout of fever. While no isolation of virus was attempted at this time, it will be noted in a subsequent paragraph that this agent circulates at least 2 days prior to the onset of the typical disease. The possibility was considered that this mild fever might represent the onset of viremia. Figures 1 and 2 show the variations in febrile response in the group of fourteen patients.

Other clinical signs and symptoms including rash. An evaluation of symptomatology in these patients was not too reliable. During the first 2 days of fever the majority complained of mild to moderate headache and aching in the back and thighs. Three individuals had mild but definite chills. There was loss of appetite, and occasional vomiting occurred. None of the patients appeared critically ill at any time, and soon after defervescence all were out of bed and had a rapid return of appetite and strength. During the course of illness, physical examination was not remarkable except for fever, splenomegaly, and rash. Three of the thirteen febrile individuals developed a definitely palpable spleen, and in two more there was questionable enlargement. A few cases developed injection of the sclerae with photophobia. There were no signs of respiratory or of meningeal involvement, and there was no significant lymphadenopathy.

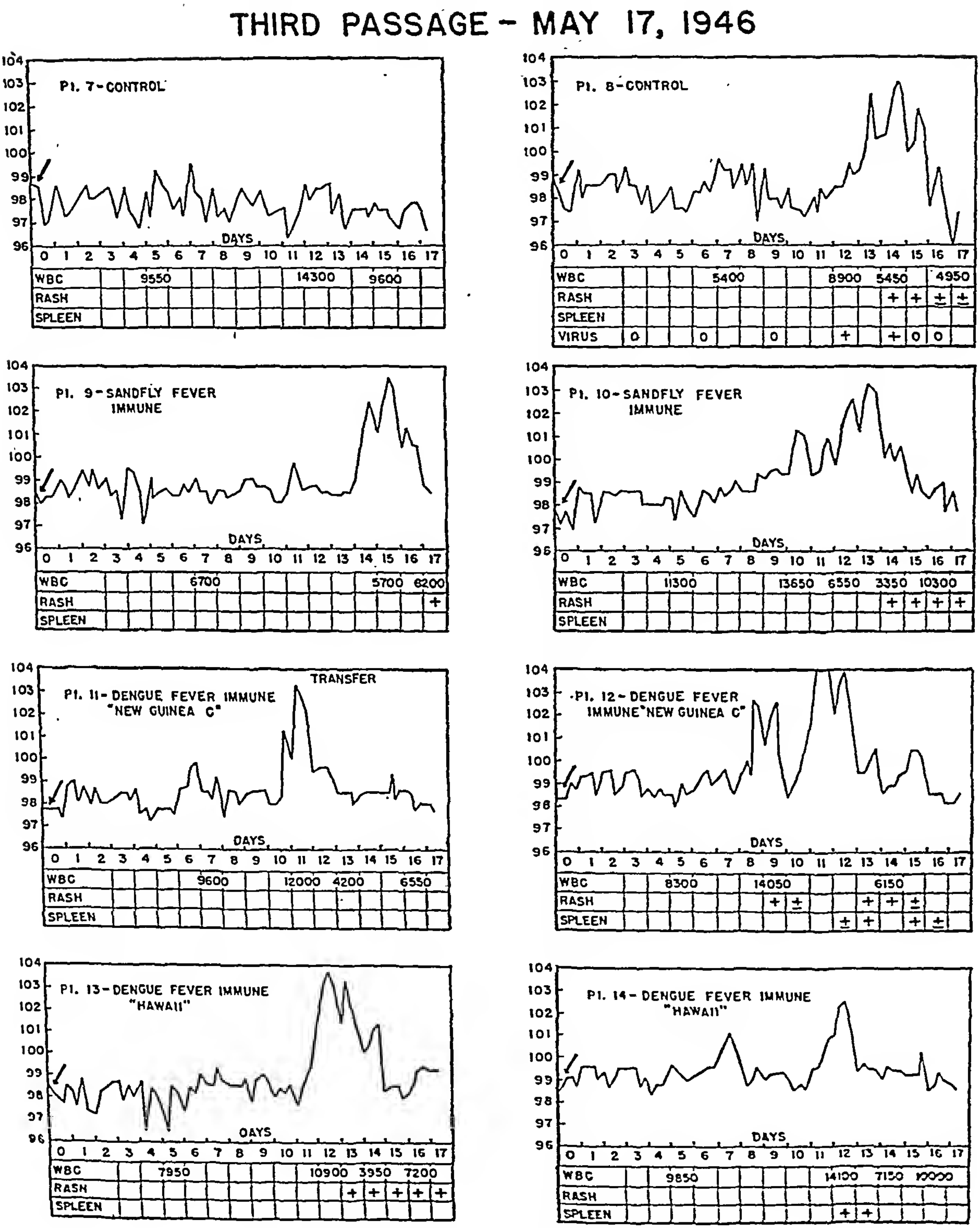
No unequivocal rash was seen in any of the patients in the first two groups. However, five of the eight members of the third group developed

[6] One additional individual, not included in the 14 mentioned above, was injected intracutaneously with only 0.4 ml. of pooled serum from the first group of patients. He failed to develop fever during the following 20 days. Whether this person, like patient 7, was resistant to the virus or whether larger doses of virus were required for transmission remained undecided.



Arrows pointing downward indicate day of inoculation
Arrows pointing upward indicate day when blood transfers were made

FIG. 1. TEMPERATURE CHARTS—EXPERIMENTAL PRETIBIAL FEVER IN MAN



Arrows pointing downward indicate day of inoculation

FIG. 2. TEMPERATURE CHARTS—EXPERIMENTAL PRETIBIAL FEVER IN MAN

TABLE II

Average total and differential leukocyte counts in the five cases with leukopenia

Days of fever	Base line*	−1**	1	2	3	4	5	6	7
Total WBC	9,531	11,775	10,950	11,017	6,500	5,400	5,940	6,775	9,400
Per cent granulocytes	62	73	79	79	64	61	55	50	65
Adult forms	57	67	73	74	52	56	43	47	61
Young forms	5	6	6	5	12	5	12	3	4
Per cent lymphocytes	36	26	20	19	34	38	43	47	27

* Average of counts taken well before onset of fever; at least 2 counts per subject.
** Indicates the day before the onset of fever; subsequent numbers indicate the days after the onset of fever.

rashes of varying extent which appeared from the second to the fifth day after the onset of fever. For the most part these were limited to the anterior and lateral surfaces of the legs. The lesions were transitory and faded rapidly after the second day with evidence of residual pigmentation and small hemorrhagic areas in two of the cases.

Some of these patients were seen by Dr. Ashley A. Weech, Professor of Pediatrics, University of Cincinnati, who described their skin lesions in this manner: "The following description of the rash in experimental pretibial fever is the result of inspecting three patients, one with presumably early lesions on the fifth febrile day, another with better lesions on the third day, and the third with lesions on the seventh day. The three cases are sufficiently similar to permit one description. Lesions were present in the skin over the lower half of the tibiae in all cases; in one subject, similar lesions were present on the dorsal surface of the left forearm. The involved areas show an erythematous blush and vary in diameter from a few millimeters to a few centimeters. The borders are well defined, but the shapes are irregular. The areas are slightly elevated above the surrounding unaffected skin and on palpation feel mildly indurated. In two lesions over the tibia of one patient there was a small ecchymosis in the center. The appearance was that of fresh hemorrhage."

In one individual (patient 12), a large erythematous blush first appeared on the right shin, followed two days later by erythema and edema at the site of intracutaneous inoculation of the virus suspension. Two days after this, maculopapular erythematous lesions appeared over both shins. None of the other cases showed any reaction at the intracutaneous inoculation site. The rashes seen in the five subjects were sufficiently similar in appearance and distribution to the rashes described in the naturally occurring disease to leave little doubt but that the clinical picture of Fort Bragg fever had been reproduced in these human subjects.

Five of the thirteen positive cases showed a slight leukocytosis with a relative increase in granulocytes which appeared late in the incubation period and persisted for a day or so after the onset of fever (see WBC in Figures 1 and 2). Beginning about the third day of the febrile phase, a slight leukopenia with a relative lymphocytosis appeared and persisted for a few days. Further interpretation of the available data did not seem warranted. Average daily values of the leukocyte counts for these five cases are shown in Table II. The hematological findings in these individuals were similar to those in patients with Fort Bragg fever.

IMMUNOLOGICAL RELATIONSHIP BETWEEN FORT BRAGG FEVER, SANDFLY FEVER, AND DENGUE FEVER

Sabin (6) found that there are distinct immunological types of dengue virus which are sufficiently related to give rise to group specific cross immunity during the first 4 to 8 weeks, although at a later date the immunity is type-specific. Similarly in sandfly fever, one strain of virus isolated from an outbreak in Italy was completely unrelated (*i.e.*, there was no cross immunity even during the first weeks) to two other viruses which were isolated in the Middle East and Sicily. The last two agents were identical in so far as yielding complete cross immunity even 2 years after a single experimental attack (6). The patients used in these tests (see Figure 2) were selected by Dr. Sabin from among those he had previously inocu-

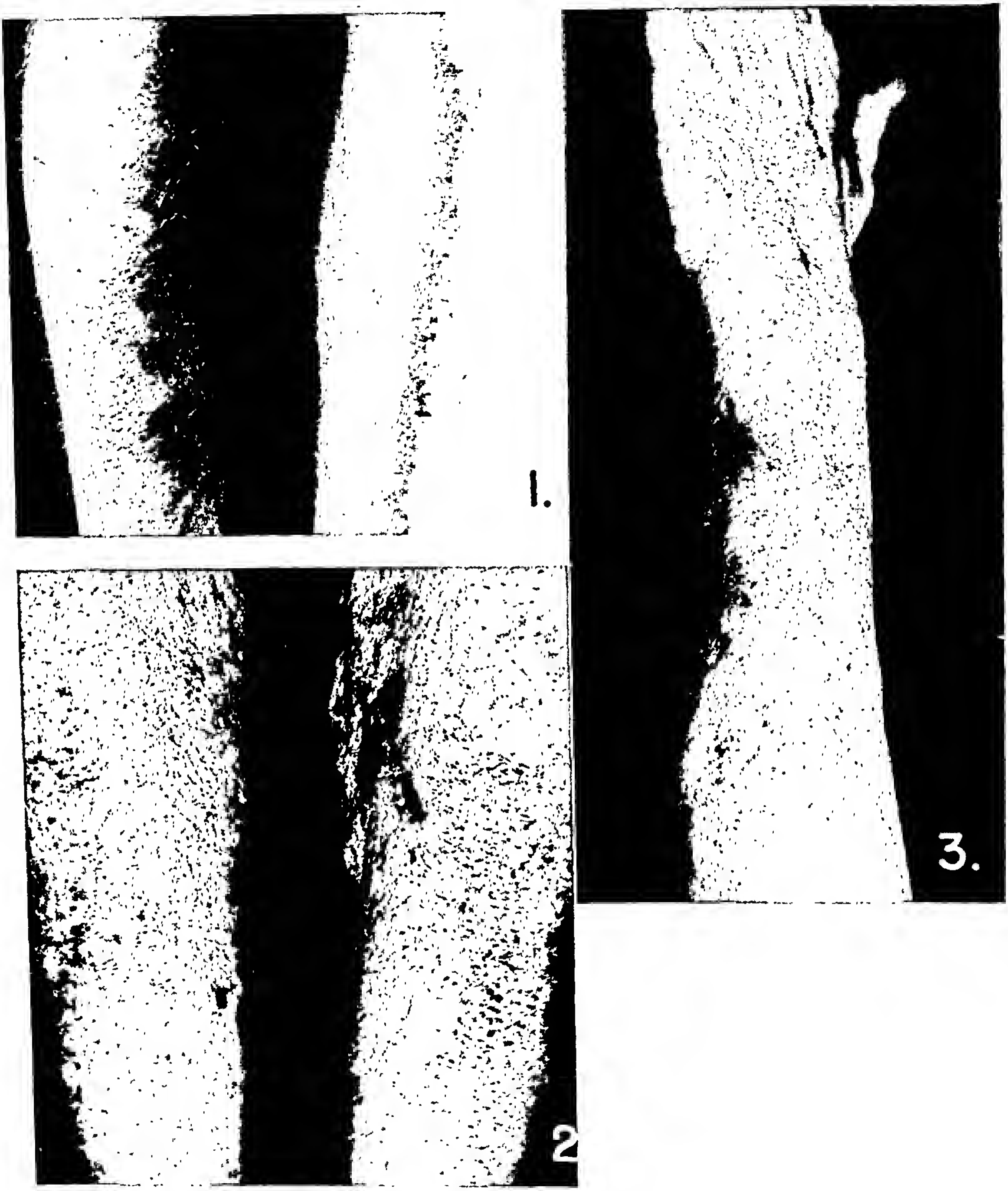


FIG. 3. ILLUSTRATION OF THE DIFFERENT TYPES OF RASH SEEN IN EXPERIMENTAL FORT BRAGG FEVER IN HUMAN BEINGS

Figures 3(*1*) and 3(*2*) show the early and late appearance, respectively, of the anterior shin lesions in patient 10. Figure 3(*3*) illustrates the raised macular lesions which appeared on the forearm of patient 13.

lated with dengue or sandfly fever viruses. Thus, patients 11 and 12, who had had typical experimental attacks of dengue following inoculation with the "New Guinea C" strain of virus 1 month before the present test, were selected since they would be expected to be immune to both "Hawaii" and "New Guinea C" strains of dengue. Patients 13 and 14, who had had experimental dengue fever following inoculation with the "Hawaii" strain of virus, 7 and 6 months, respectively, prior to the present test, would resist only an agent immunologically identical with the "Hawaii" type of virus. Similarly, patients 9 and 10 could indicate a relationship only between the new agent and the "Middle East—Sicilian" type of sandfly fever virus (5) but not with sandfly fever in general. Patient 9 had had his first attack in November, 1943 (2½ years prior to the present test) and was immune when challenged with the homologous virus in October, 1945. Patient 10 had had his experimental attack in October 1945, or 7 months before the present test. Since all six patients developed the experimental disease produced by the new agent, it can be concluded that it possesses neither a group relationship with the known dengue viruses nor any relationship with one strain of sandfly fever virus. It may be stressed, furthermore, that neither the dengue nor the sandfly fever viruses possess the pathogenic properties of the new agent for hamsters and guinea pigs.

DETECTION OF VIRUS IN THE BLOOD OF PATIENTS

Information on the time of appearance and duration of viremia in experimentally infected human subjects was obtained in the following manner. Blood was drawn from the patients at various times during the disease, immediately defibrinated, and injected into young hamsters. In certain instances the animals received 2.0 ml. of undiluted blood intraperitoneally. In others, serial tenfold dilutions of serum were injected intracerebrally in order to determine the infective titer. Those hamsters which died between the seventh and sixteenth days were assumed to have succumbed to infection with the virus under investigation. The results of these tests in patients 1, 2, and 3, Figure 1, clearly showed that virus was present at least 48 hours before the onset of fever and was still detectable on the second day of fever, but not on the fourth day or thereafter. Patient 8, Figure 2, had detectable virus circulating 24 hours before and 24 hours after the onset of fever, but not 4 days before nor 3 days after the onset of fever. In two instances, undiluted blood from patient 8 was lethal for hamsters but failed to kill in dilutions of 1:10 or greater. It may be concluded that the virus appeared in the blood of these patients shortly before the onset of fever and disappeared promptly; furthermore, it did not occur in large amounts.

SUMMARY AND CONCLUSIONS

An infectious agent was recovered from guinea pigs injected with blood from a soldier suffering from Fort Bragg fever in August, 1944 at Fort Bragg. The agent induced a febrile non-fatal illness in guinea pigs and rabbits and a lethal disease in hamsters. It was maintained by passage in animals or embryonated eggs, but storage even at − 70° C. was unsatisfactory unless special precautions were taken. The agent was filterable under conditions which were adequate to retain staphylococci.

The virus, after prolonged passage in guinea pigs and embryonated eggs, was able to induce the clinical picture of Fort Bragg fever in some of the inoculated human beings, while the majority exhibited only fever for 1 to 3 days.

The new virus appears to be unrelated in its properties or immunologically to the agents of lymphocytic choriomeningitis, Q fever, Rocky Mountain spotted fever, sandfly fever, and dengue fever. It does not resemble the rickettsia-like agent previously encountered in work on Fort Bragg fever and subsequently shown to be enzootic in guinea pigs.

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BIBLIOGRAPHY

1. Daniels, W. B., and Grennan, H. A., Pretibial fever, an obscure disease. J. A. M. A., 1943, 122, 361.
2. Topping, N. H., Philip, C. B., and Paul, J. R., Report of the Commission for the Study of an Unidentified Disease at Fort Bragg, N. C. (Sept. 3, 1942–Sept. 11, 1942). Preliminary report submitted to the Surgeon General, U. S. Army, 15 Oct. 1942; final report 31 March 1943.
3. Commission on Acute Respiratory Diseases, Identification and characteristics of the Balkan grippe strain of *Rickettsia burneti*. Amer. J. Hyg., 1946, 44, 110.
4. Tatlock, H., A rickettsia-like organism recovered from guinea pigs. Proc. Soc. Exper. Biol. & Med., 1944, 57, 95.
5. Sabin, A. B., Philip, C. B., and Paul, J. R., Phlebotomus (Pappataci or sandfly) fever, a disease of military importance, summary of existing knowledge and preliminary report of original investigations. J. A. M. A., 1944, 125, 603, 693.
6. Sabin, A. B. Personal communication.

ELECTROPHORETIC CHANGES IN THE SERUM PROTEIN PATTERN OF A PATIENT WITH TYPHUS FEVER[1]

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In an earlier communication (1) attention was called to the marked depressions of albumin: globulin ratios that frequently occur in the course of louse-borne typhus fever. Serum globulin concentrations in a number of cases were found to be higher than 5 grams per 100 ml. Because of the consistent rise in serum globulins in this disease, possibly a feature of the immune response, it was of interest to determine which globulin components were increased and to observe the sequence of changes associated with the clinical course.

The electrophoretic analyses to be described were made on serum samples taken from a patient with clinically typical severe typhus fever, included in the group previously reported. It will be seen that the depression of his albumin: globulin ratio was associated with a striking increase in the γ globulin component.

PROCEDURE

Sera were stored in sterile tubes at 4° C. without preservative. After the collections were complete they were brought from Egypt to New York City, refrigerated in transit.

The electrophoretic analyses were made in diethylbarbituric acid buffer (μ 0.1, pH 8.6) as previously described (2).

OBSERVATIONS AND DISCUSSION

A. S. M. (No. 2501), a 21-year-old Egyptian man, was admitted to the Typhus Commission ward on the third day of disease, acutely ill. There was no history of previous typhus, nor had he received anti-typhus vaccine.

During the first three weeks of hospitalization he had a sustained high fever. Delirium, vomiting, and a typical rash were present during the first week; intractable diarrhea during the second, and attacks of bronchial asthma during the third. In the latter part of his febrile course, dysarthria appeared with periods of emotional depression, increased tendon reflexes, spasticity of extremities, and adiadokokinesis. These neurological signs gradually disappeared during the subsequent five weeks of convalescence. At the time of discharge on the fifty-ninth day of disease, he was underweight, but the physical examination in other respects was normal.

On the fifth day of disease the Weil-Felix and complement fixation reactions were negative. On the forty-seventh day these tests were positive in dilutions of 1/320 and 1/1280 respectively.

OBSERVATIONS AND DISCUSSION

The results of electrophoretic analysis and the associated clinical data are charted in Figure 1.

The total protein, low during the early febrile stage, rose to abnormally high values in convalescence.

The relative proportion of albumin, and with it the albumin to globulin ratio, was found to be markedly depressed even on the fourth day of disease. Similar, but lesser, early depressions of albumin have been observed in patients with malaria (3) and with scarlet fever (4). Presumably this is a non-specific reaction to an acute febrile illness.

The relative proportion of α_1 globulin was not significantly disturbed; α_2 globulin showed a slight sustained increase. The practical absence of change in these components contrasts with the conspicuous increase found in patients with scarlet fever (4).

β globulin was on the low side of normal throughout the illness.

Most interesting is the early marked elevation of γ globulin. If it be assumed that the patient had a normal distribution of serum protein components prior to illness, this phenomenon would appear to indicate that there is a considerable release of γ globulin protein during the acute reaction to typhus infection. It would seem unlikely that the increase in γ globulin on the fourth day of disease could have been due to production of

[1] The Bureau of Medicine and Surgery does not necessarily endorse opinions expressed in this paper.

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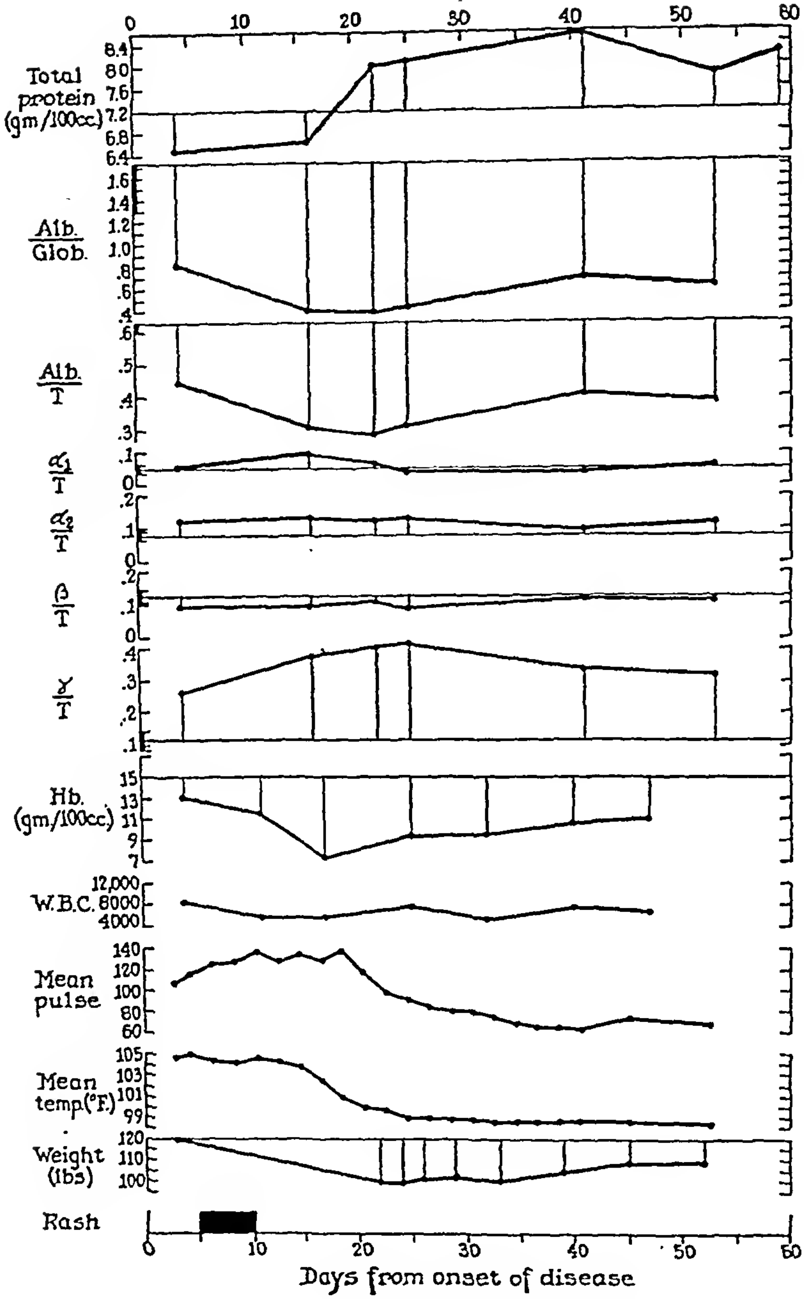

FIG. 1. ELECTROPHORETIC AND CLINICAL DATA DURING THE COURSE OF TYPHUS FEVER

From above downwards the ordinates show total protein concentration, the albumin: globulin ratio (measured electrophoretically), the fraction of the total protein (T) included in the different electrophoretic components, and the clinical data indicated. Horizontal lines show normal values, with the range of one standard deviation about the normal indicated by the heavy vertical brackets.

specific antibodies at that early date in a person without previous exposure to the antigens concerned. The available data, moreover, indicate that at least some of the antibodies which appear in the course of typhus fever were not then present, since the Weil-Felix and complement fixation tests were negative at that time, although later positive in high dilutions. It would further appear probable that protective antibodies were not present in significant concentration on the fourth day since the patient continued to be severely ill for over two weeks subsequently.

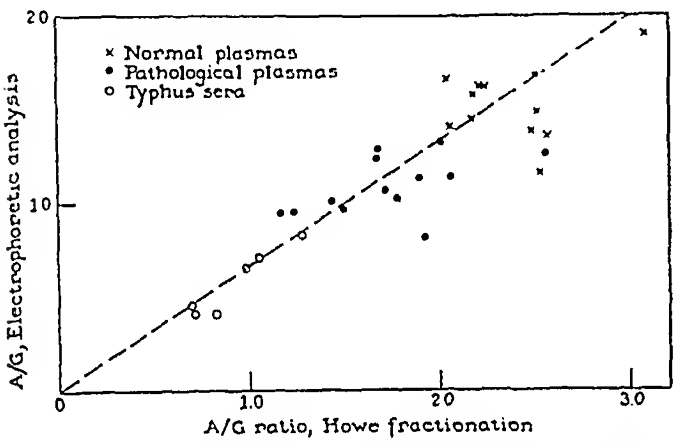

FIG. 2. CORRELATION OF ALBUMIN: GLOBULIN RATIOS MEASURED BY ELECTROPHORETIC ANALYSIS AND BY HOWE SODIUM SULFATE FRACTIONATION

Open circles show the new data.

The exceptionally low albumin: globulin ratios encountered in these sera presented an opportunity to extend the previously reported (2) correlation between the ratios determined by electrophoretic analysis and by the customary Howe sodium sulfate fractionation. It may be observed in Figure 2 that the newer data (open circles) approximate the same line drawn to fit earlier values. It would thus appear proper to infer from the more extensive study on typhus fever serum proteins (1) that the marked depressions observed in albumin: globulin ratios by salt fractionation would imply comparable abnormalities in the electrophoretic ratios.

SUMMARY

1. The relative proportion of albumin and the albumin: globulin ratio were markedly reduced on the fourth day of typhus fever and through the convalescence.

2. The α and β globulins were practically unaffected.

3. γ globulin was increased to a striking degree on the fourth day and to an even greater extent in convalescence.

4. A previously described relation between the albumin: globulin ratios determined electrophoretically and by salt fractionation was found to remain valid for the lower ratios encountered in this study.

BIBLIOGRAPHY

1. Tierney, N. A., and Yeomans, A., Metabolic studies in louse-borne typhus. Observations on serum electrolyte pattern, serum protein partition, and nitrogen balance. J. Clin. Invest., 1946, 25, 822.
2. Dole, V. P., The electrophoretic patterns of normal plasma. J. Clin. Invest., 1944, 23, 708.
3. Dole, V. P., and Emerson, K., Electrophoretic changes in the plasma protein patterns of patients with relapsing malaria. J. Clin. Invest., 1945, 24, 644.
4. Dole, V. P., Watson, R. F., and Rothbard, S., Electrophoretic changes in the serum protein patterns of patients with scarlet fever and rheumatic fever. J. Clin. Invest., 1945, 24, 648.

THE ABSORPTION, EXCRETION, AND PHYSIOLOGICAL EFFECT OF IODINE IN NORMAL HUMAN SUBJECTS

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Although iodine has been used as a therapeutic agent for many years, information concerning its absorption, excretion, and physiological effect in normal men is not extensive. Several investigators have studied the uptake of iodine in the normal and pathological thyroid (1, 2, 3, 4, 5) and others, in balance studies, have sought to measure the daily iodine requirement of the body as a whole (6, 7, 8, 9). In general, very small amounts of iodine were used in these studies and the results cannot be applied readily to the fate of large quantities of iodine. This study deals with the absorption and excretion of iodine in quantities ranging from 20 mgm. to 440 mgm. per day, more than a thousand times the estimated daily requirement (4, 10), but within the range of clinical administration for syphilis, roentgenography, and for use as an expectorant, and only slightly above the commonly administered dosage of Lugol's solution for the treatment of thyroid disorders (11).

Two toxic effects of iodine in normal human subjects are well known, the direct toxic action of free iodine in aqueous or alcoholic solution on tissue protoplasm, and iodism or iodine sensitiveness, occurring in a small percentage of cases (12, 13, 14). It was formerly thought that iodine could give rise at times to hyperthyroidism (15, 16, 17), but more recently the consensus is that iodine has no effect on the basal metabolic rate of normal persons (4, 18, 19, 20, 21). Studies in this regard, however, have been limited. The few subjects used were not closely observed or were not followed for more than one or two weeks. Iodized salt has been used in large population groups without ill effect (22) but the amount of the halogen ingested daily was very small. In rabbits, a fall in metabolism due to Lugol's solution has been noted, reaching minimum values on the third day, and followed by a return to normal (23). Other animal studies have been equivocal in this regard (24, 25, 26, 27, 28). Iodine has been thought to increase nitrogen excretion in dogs and in man. (29).

In the present study a group of 13 healthy soldiers ingested large quantities of water containing an average of 34.8 mgm. per liter of iodine over a period of 38 days.[1] Iodine absorption from the gastrointestinal tract, excretion in the urine, sweat, and feces, and the concentration of iodine in the plasma were determined. A rough balance study was possible. The subjects worked and lived throughout the test in a hot humid environment. This led to the consumption of large amounts of iodine containing water and facilitated the study of iodine excretion in the sweat. The men were subjected to severe physical stress and observations were made on general health, work performance, acclimatization to heat, and basal metabolic rate.

METHODS

Experimental conditions. The subjects were divided into groups A and B, of 3 and 10 men respectively, on the basis of physical characteristics and work performance during a preliminary control period of 6 days. The environmental temperature at this time was kept at 80 degrees F. dry bulb and 30 to 50 per cent relative humidity. The test period in which iodine was administered followed immediately and lasted 45 days. During this time the environment of the laboratory room was maintained at a dry bulb temperature of 90 to 91 degrees F. and 95 per cent relative humidity from 8:00 A.M. to 4:30 P.M., but was returned to the relatively cool environment of the control period in the remaining hours of the day and night. The test period was followed by a final control

[1] This report is derived from a study of Bursoline, an iodine-containing agent for the disinfection of water. A Bursoline tablet contains 8.2 mgm. of free iodine and 8.2 mgm. of iodine combined as diglycine hydroiodide. On addition to water Bursoline yields a solution of free and ionic iodine.

period of 3 days, identical with the first period. All men, unless incapacitated, walked 5 hours each day except Sunday, 3 hours in the morning and 2 hours in the afternoon, at an average pace of 2.8 miles per hour. They wore shorts and regulation army shoes only, and carried 20-pound packs. They remained in the laboratory room day and night.

Group B drank iodine-containing water in the first 38 days of heat exposure while group A drank salted tap water. During the last 6 days of heat exposure, group A drank iodine-containing water and group B drank salted tap water (Table I). On Sundays when the men did not work, water requirements were low and the iodine ingestion consequently was reduced.

The final iodine content of the drinking water averaged 34.8 mgm. per liter of which 34 per cent was free iodine and 66 per cent iodide.

TABLE I

Regimen of exposure to iodine and heat

	Cool control 6 days	Iodine and moist heat 45 days			Cool control 3 days
	Days 6 through 1	Days 1 through 38	Day 39	Days 40 through 45	Days 46 through 48
Group A	T	S	S	I & S	T
Group B	T	I & S	S	S	T

T = Tap water, untreated.
S = Tap water, salted to 0.1 per cent.
I & S = Iodine water, salted to 0.1 per cent.

Experimental procedures. All water was dispensed by a trained technician and the consumption recorded. Twenty-four hour urine collections were made on all subjects throughout the study. During the period of iodine intake, blood samples were obtained on Monday morning and afternoon,

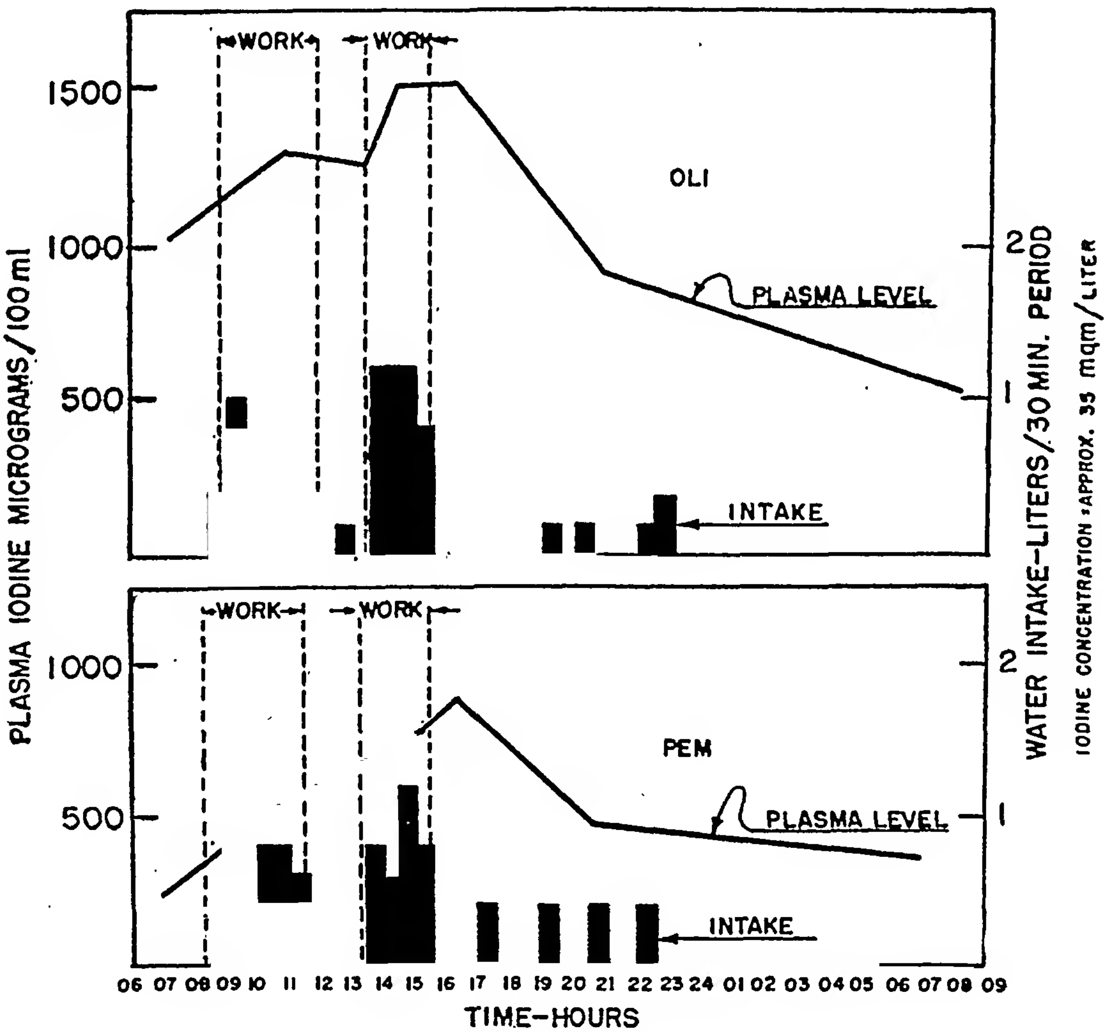

FIG. 1. PLASMA IODINE VERSUS IODINE WATER INTAKE

Wednesday afternoon, and Saturady morning and afternoon. After stopping iodine, only morning specimens were collected. Sweat collections were made at intervals by using dry suits of clothing for total sweat, or arm length rubber gauntlets for arm sweat. Feces were collected from 2 men for 7 consecutive days.

Details of the chemical methods will be given in a separate report (30).

Plasma rather than whole blood was chosen for analysis because of the simplified technic of determination and the expectation that, since the distribution of the iodide ion is similar to that of chloride (31), the plasma would give a more stable index of iodide concentration than whole blood. In addition, since the protein bound and organic iodine are removed with the protein precipitate (32, 33) a protein-free filtrate provided a homogeneous source of iodide ion. The available evidence (33) indicates that ingestion of iodine or iodide primarily influences the ionic iodide fraction of plasma. Iodine present in body fluids is believed to exist exclusively in the reduced form. This is suggested both by the redox potential of these fluids and by demonstration (34) that iodine is absorbed only after reduction to iodide. Unless iodine or iodides are being taken, only minute amounts of ionic iodide are found in plasma (4), certainly less than 3 or 4 μg. per 100 ml. which is the lower limit of detection by the method of analysis used here.

During the 38 days in which Group A drank salted tap water, the plasma collected from these men was uniformly negative for iodine (less than 10 μg. per 100 ml.); in four instances small amounts of iodine were found in the urine (probably contamination), but otherwise all were negative.

Observations were made on flushing, vigor, alertness, and the general reaction of subjects to work in the heat. Symptoms of headache, dizziness, nausea, palpitation, and gastrointestinal cramps were noted. Pulse rates and rectal temperatures were taken before, during, and after the periods of work. Sweat loss was calculated from the weight difference between the beginning and end of work periods, corrected for water intake, urine output, and sweat unevaporated at the time of weighings. Blood pressures were noted at frequent intervals.

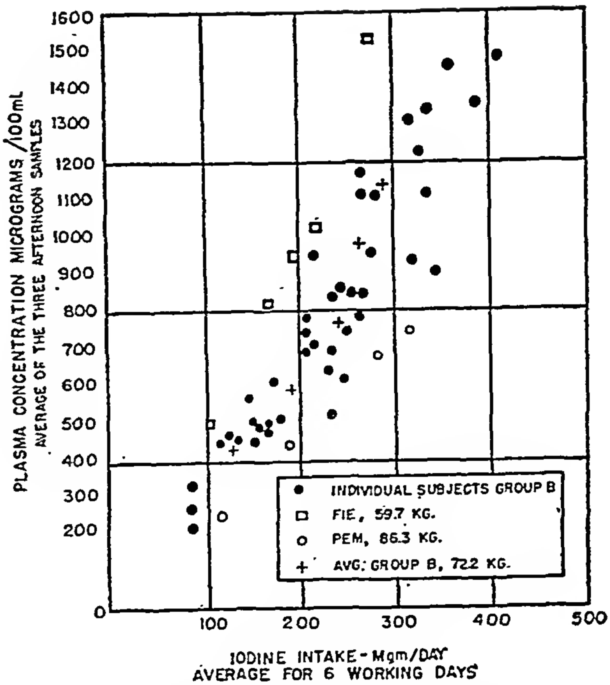

FIG. 2. RELATIONSHIP BETWEEN IODINE INTAKE AND PLASMA IODINE CONCENTRATION (GROUP B)

Basal metabolism was measured by the closed circuit Sanborn Waterless and Benedict-Roth machines. The tests were made before 8:00 A.M. when the subjects with rare exceptions had not moved from their cots. Twenty determinations were made on each subject, 13 of them in the period of exposure to iodine and heat.

RESULTS

Absorption of iodine. Iodine was rapidly absorbed from the gastrointestinal tract. This is apparent in Figure 1 in which iodine ingested and plasma iodine concentrations are plotted as functions of time. A rise of plasma level occurred after each period of iodine intake. The dip in the climbing portion of the plasma concentration curve which appeared in subject Oli indicated that within a period of 1 to 2 hours after cessation of intake the peak of absorption had been passed. Absorption was complete since no significant quantity of iodine was recovered from the stools of the subjects on high intakes. The level of plasma iodine was directly related to the intake as is best seen in Figure 2.

This proportionality over longer periods of time appears in Figure 3 where plasma concentrations

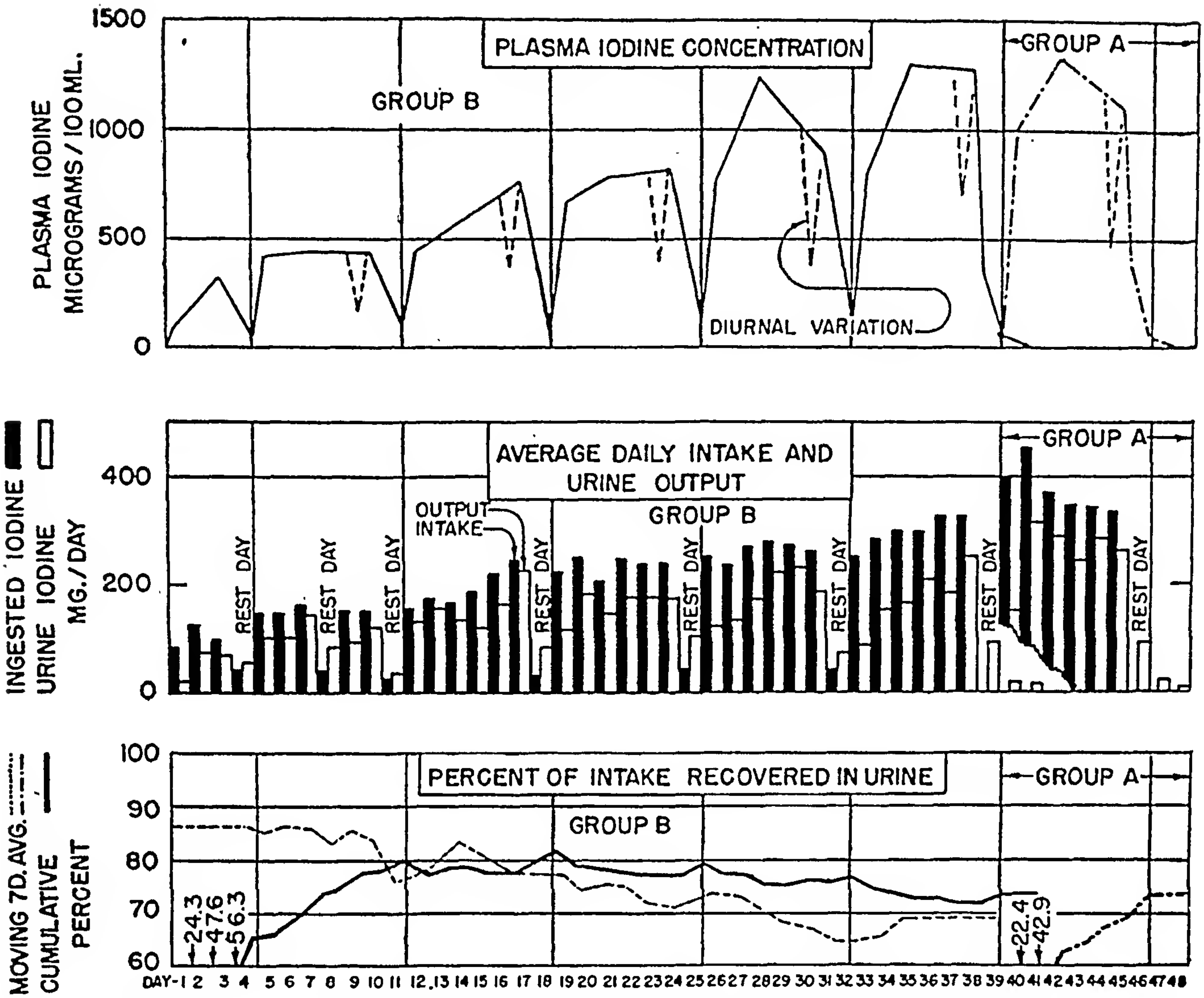

FIG. 3. PLASMA IODINE CONCENTRATION, IODINE INTAKE, AND URINE OUTPUT (GROUP A—AVERAGE OF 3 MEN; GROUP B—AVERAGE OF 10 MEN)

are shown in the upper panel, and intakes, plotted as solid columns for individual days, appear in the middle panel. The broken lines extending as V's from the solid envelope of plasma concentrations indicate the extent of diurnal variation.

Excretion of iodine. The uniformity and rapidity of the disappearance of iodine from the plasma are indicated in Figure 4 where iodine level is plotted on semilogarithmic scale against time. The linear plots suggest that a uniform rate of disappearance, proportional to the concentration, prevails during the elimination of iodine. From the slope of the average line, it is seen that if no more iodine is taken, plasma iodine concentration falls by 83 per cent (to 17 per cent of its initial value) in 24 hours.

The chief route of iodine elimination was in the urine. As shown by the solid line in the lower panel of Figure 3, 74 per cent of all iodine given was recovered in the urine in Group B and the same percentage subsequently in Group A. In each man, excretion in considerable amounts continued through the third day after cessation of intake, but only in insignificant quantities thereafter. This delay in excretion prevented a useful calculation of daily yield, for, on the rest day of each week, in which little iodine was taken, there occurred nevertheless a fairly large iodine excretion (Figure 3). This lag was due to the iodine content of the extracellular fluid. To compensate for the lag, the device of calculating urine return in terms of a moving 7-day average was used.

Thus in each 7-day average the excretion from 1 day in which the intake was low was included. This average yield is shown on the bottom panel of Figure 3 as a broken line. The decrease in per cent recovered in the urine which is apparent can be correlated with the increase in sweat output (weight loss) over the experimental period (Figure 5), and reflects the increased loss of iodine in sweat at high sweat outputs.

An attempt was made to obtain short-time measurements of the rate of iodine excretion in the urine in order to define the characteristics of urinary excretion as a function of plasma level and other factors. However, the tendency of all subjects to lag behind their water requirements during the period of active sweating led to low rates of urine flow and made accurately timed collections of urine very difficult. The available data indicated clearance rates from 10 to 25 ml. of plasma per minute with a tendency toward higher rates with increasing urine flow. Such clearances are consistent with the slopes of the plasma iodine disappearance lines in Figure 4.

The sweat was of next importance in the elimination of iodine. In Figure 6 it is apparent that sweat iodine concentration is related to plasma iodine concentration; roughly, the concentration of sweat iodine is 35 per cent of the concentration of plasma iodine. It can be seen that while the iodine level in arm sweat was of the same order of magnitude as the whole body sweat, it was nevertheless uniformly higher in relation to the

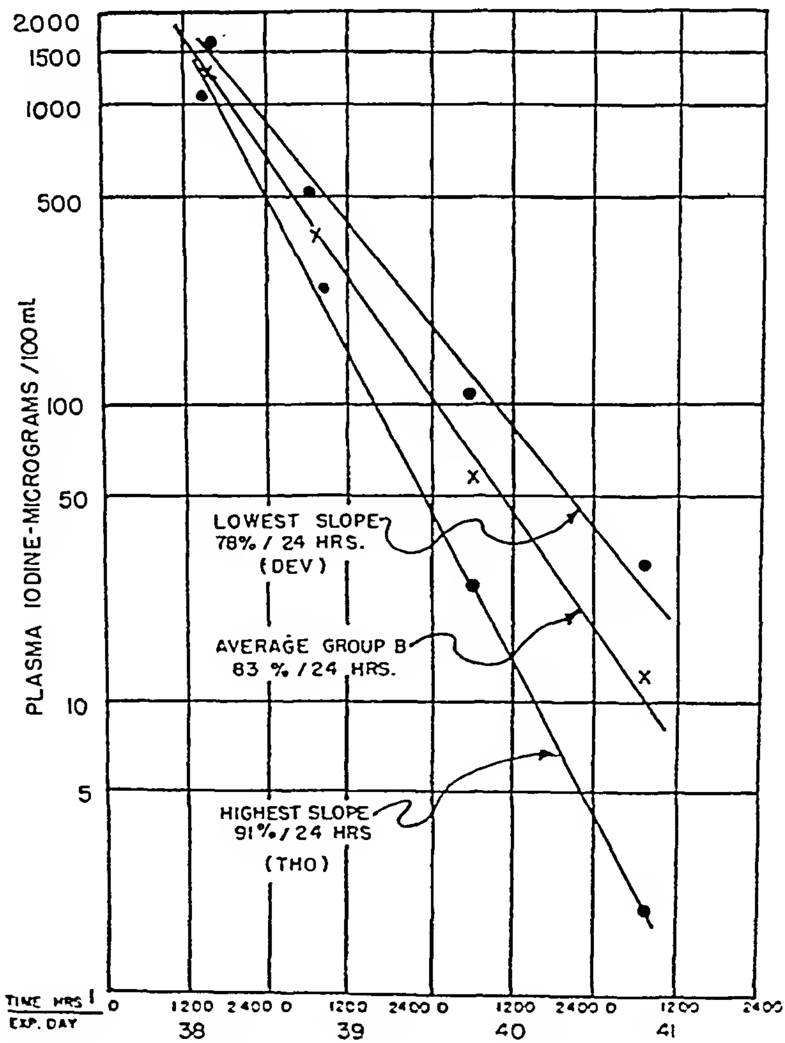

FIG. 4. RATE OF DISAPPEARANCE OF IODINE FROM THE PLASMA (GROUP B)

plasma level than whole body sweat by 25 per cent to 30 per cent. This may be due in part to regional differences in concentration.

Data pertinent to the question of analogy in sweat excretion between chloride and iodide were obtained in two experiments each on subjects Del and Bur. The results of these experiments are given in Table II. It appears that the concentration of both iodide and chloride in the sweat depends on the plasma concentrations in much the same way.

Relative to the amount ingested, only minor

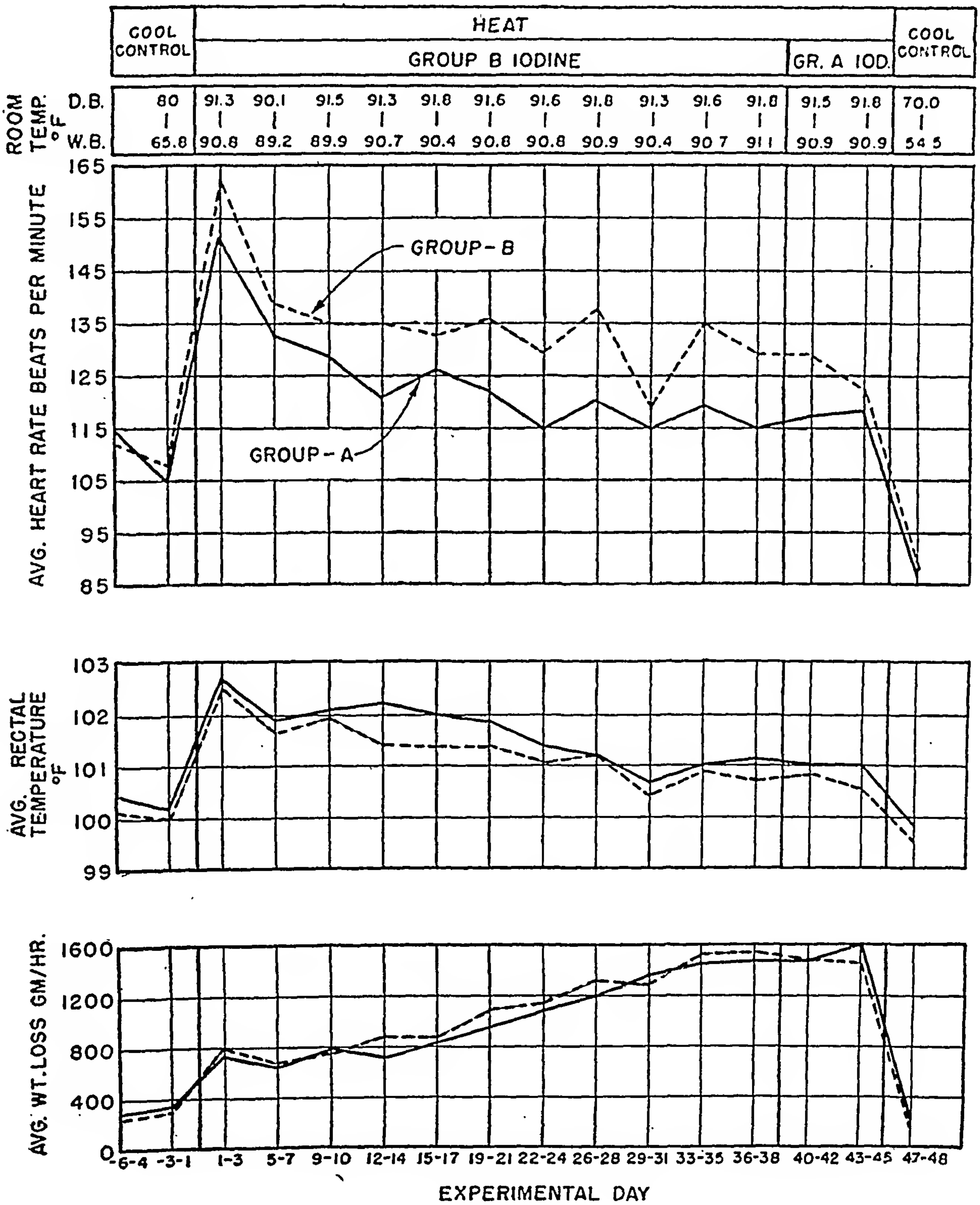

FIG. 5. HEART RATE, RECTAL TEMPERATURE, AND HOURLY WEIGHT LOSS BY 3-DAY PERIODS

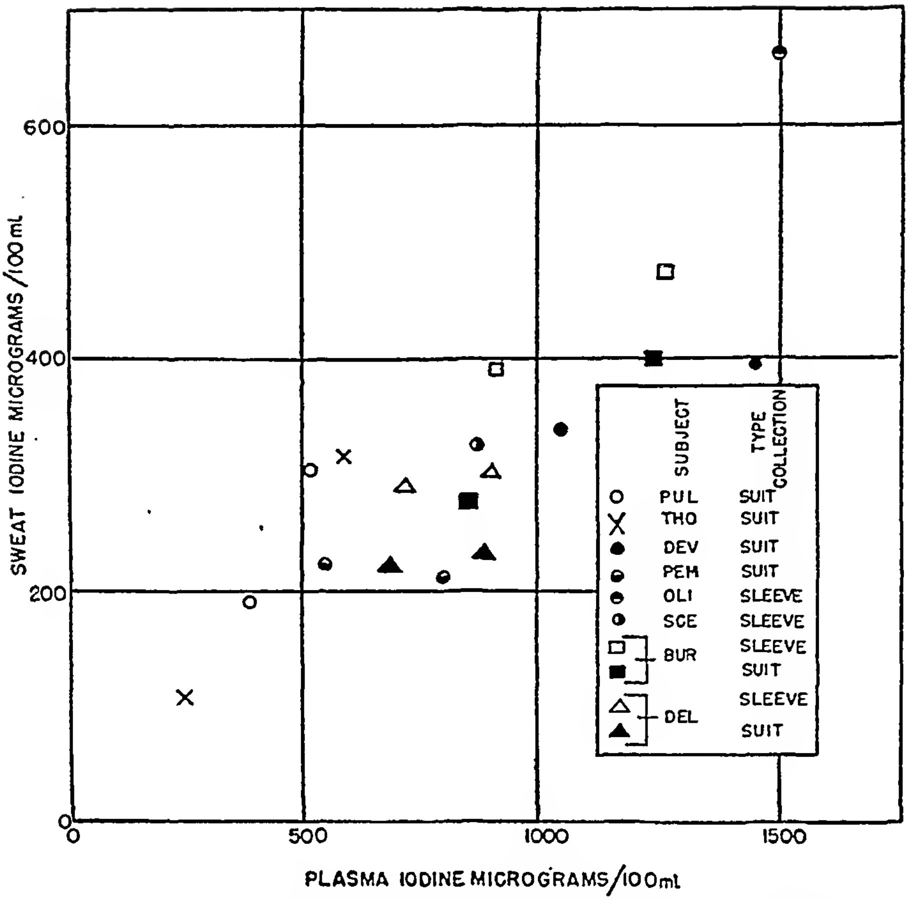

FIG. 6. CONCENTRATION OF IODINE IN SWEAT AS A FUNCTION OF PLASMA IODINE CONCENTRATION

amounts of iodine were found in the feces. Complete collections during 1 week made on subjects Pul and Tho led to recoveries of 0.32 per cent and 0.38 per cent respectively of ingested iodine.

Iodine balance. With the information available on the urine, sweat, and feces, it was possible to assemble the partial balance shown in Table III. The basis of calculation of the various components was as follows: the intake and urine output were taken from the sources already mentioned; sweat iodine was calculated from the total sweat excretion and an estimated iodine concentration derived from the correlation in Figure 6 (Sweat I = 0.35 plasma I, where plasma I was taken as 0.77[5] average peak plasma concentration); fecal output was calculated as a percentage of the total intake based on the results from the subjects Tho and Pul of 0.38 per cent and 0.32 per cent respectively.

The resulting balance, though based on crude estimates, gives a fair picture of the relative importance of the various paths of iodine loss under the circumstances of this experiment.

TABLE II

Iodide and chloride excretion in the sweat as a function of plasma concentration

Arm sweat collected for 40-minute period; plasma values interpolated to midpoint of period

Subject	Iodide			Chloride		
	Plasma	Sweat	Ratio I_s/I_p	Plasma	Sweat	Ratio Cl_s/Cl_p
	µg. per l.	*µg. per l.*		*meq. per l.*	*meq. per l.*	
Bur 1	910	3.94	0.433	95.5	41.5	0.434
2	1,260	4.75	0.377	93.5	34.5	0.369
Del 1	720	2.95	0.410	101.7	36.8	0.362
2	900	3.05	0.339	98.7	29.2	0.296

[5] The factor 0.77 was derived from curves of the type shown in Figure 1 to approximate an integrated average concentration for the work periods, the time of high sweat output.

TABLE III

Approximate iodine balance

(See text for methods of estimation)

Subject	Week	Iodine balance								
		In	Out							
			Urine		Sweat		Feces		Total	
		mgm.	*mgm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*
SCE	2	494	431	87.3	11	2.3	1.7	0.35	444	89.9
	5	1,675	1,475	88.0	69	4.1	5.9	0.35	1,550	92.5
	All	5,677	4,984	87.8	215	3.8	19.9	0.35	5,218	91.9
THO	2	550	450	81.8	17	3.0	1.9	0.35	469	85.1
	5	1,225	1,008	82.3	79	6.5	4.3	0.35	1,092	89.1
	All	5,699	4,609	80.9	337	5.9	20.0	0.35	4,966	87.1
DEV	2	895	690	77.1	37	4.2	3.1	0.35	730	81.6
	5	1,623	1,197	73.8	153	9.4	5.7	0.35	1,356	83.5
	All	7,460	4,904	65.7	575	7.7	26.1	0.35	5,504	73.8
Group B (10 Men)	2	804	691	85.9	24	3.0	2.8	0.35	718	89.3
	5	1,621	1,157	71.4	117	7.2	5.7	0.35	1,280	78.9
	All	7,143	5,283	73.9	430	6.0	25.0	0.35	5,738	80.3

Physiological effects. The subjects ingested an average of 88 mgm. of iodine daily and attained blood plasma concentrations ranging from 75 to 1,300 μg. per 100 ml. during the 38-day period of exposure (Figure 3).

No deleterious effect on performance of work and no unusual hardships during acclimatization to heat were noted in those men taking iodine when compared with the control group or in comparison with other groups of normal subjects under similar conditions of stress (35, 36). The measurement of final postwork heart rate, rectal temperatures, and hourly sweat rates for the control and test periods are presented in Figure 5. It is apparent that the responses of both groups of men were parallel, regardless of the intake of iodine.

The results of the basal metabolism tests are presented in Figure 7. The group averages showed no trend, nor was any trend noted in the analysis of the results of individual subjects. No apparent change in basal metabolic rate due to heat exposure alone under the conditions of this experiment could be found. No change was observed in the size or consistency of the thyroid gland, and the neck circumference did not alter. There was an insignificant weight loss in both groups.

Blood and urine examinations before and after exposure to heat and iodine revealed only a slight fall in blood hemoglobin, similar to that noted in other groups of subjects exposed to heat alone.

The general symptomatology of the men in both groups was similiar and was that of acclimatization as has been described elsewhere (35, 36). Blood pressure measurements were not remarkable. Of

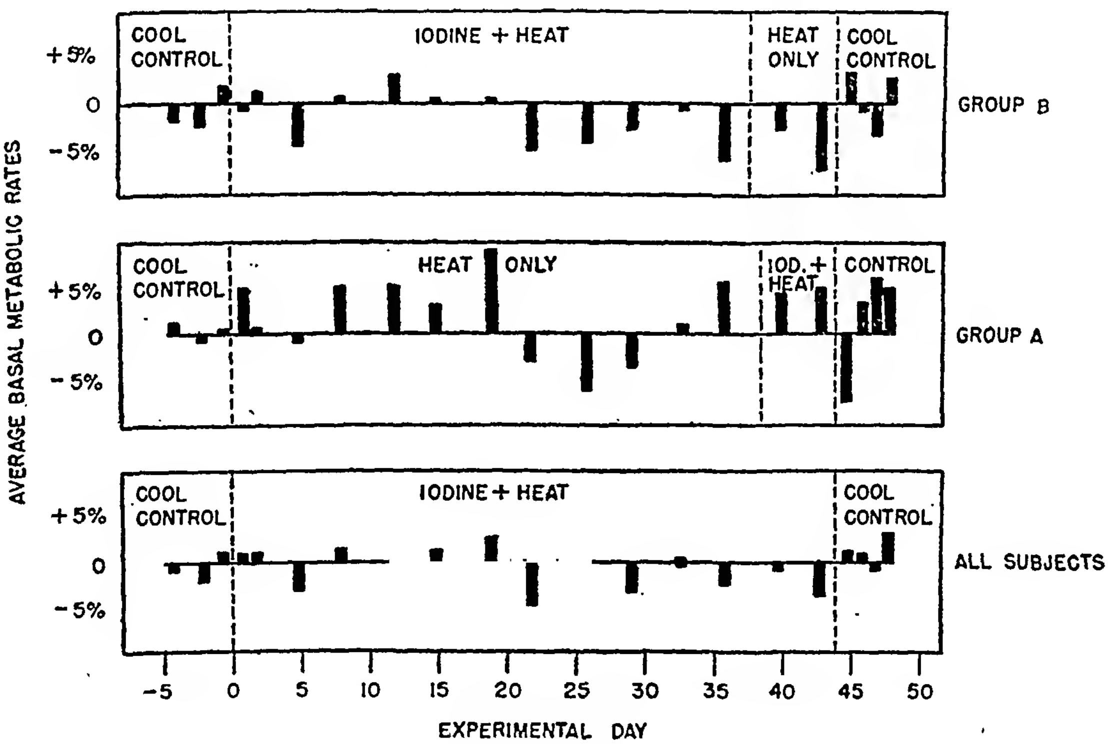

FIG. 7. EFFECT OF IODINE AND HEAT ON THE BASAL METABOLIC RATE

incidental interest was the fact that the taste of iodine water, found quite disagreeable at first, was preferred by the subjects to salted tap water at the end of the period of exposure.

The men were tested for skin sensitivity to iodine during and at the conclusion of the experiment but the small number of subjects precluded drawing any useful conclusions in that regard.

SUMMARY

(1) Inorganic iodine was rapidly and completely absorbed from the gastrointestinal tract.

(2) The plasma concentration of iodine was directly proportional to the quantity ingested.

(3) The iodine plasma level fell rapidly after cessation of iodine intake reaching near basal levels within 72 hours.

(4) Excretion of iodine was chiefly in the urine but an appreciable fraction appeared in the sweat. Sweat iodine concentration was 35 per cent of plasma concentration. Fecal excretion was negligible.

(5) No effect on general health, acclimatization to heat, vital signs, basal metabolism, blood, or urine could be ascribed to the ingestion of iodine by normal subjects over a period of 6 weeks.

We wish to thank T. C. Swigert, J. L. Gray, R. J. Bloom, and F. W. Urbush for technical assistance.

BIBLIOGRAPHY

1. Cole, V. V., and Curtis, G. M., Iodine metabolism of adult rat in relation to trauma, thyroid activity and diet. J. Pharmacol. & Eper. Therap., 1936, 56, 351.
2. Elmer, A. W., Iodine Metabolism and Thyroid Function. London, Oxford University Press, Humphrey Milford, 1938.
3. Mann, W., Leblond, C. P., and Warren, S. L., Iodine metabolism of the thyroid gland. J. Biol. Chem., 1942, 142, 905.
4. Salter, W. T., The Endocrine Function of Iodine. Cambridge, Mass., Harvard University Press, 1940.
5. Hamilton, J. G., Rates of absorption of radioactive isotopes of sodium, potassium, chlorine, bromine, and iodine in normal human subjects. Am. J. Physiol., 1938, 124, 667.
6. Orr, J. B., and Leitch, I., Iodine in Nutrition. A Review of Existing Information. Med. Res. Council Special Report, Series 123. London, 1929.
7. Cole, V. V., and Curtis, G. M., Human iodine balance. J. Nutrition, 1935, 10, 493.
8. Fellenberg, T. von, Research on iodine metabolism. Biochem. Ztschr., 1926, 174, 341.
9. Scheffer, L., Jodgehalt der Nahrung und Kropfhäufigkeit. Biochem. Ztschr., 1932, 247, 418.
10. Rawson, R. W., Moore, F. D., Peacock, W., Means, J. H., Cope, O., and Riddell, C. B., Effect of iodine on the thyroid gland in Graves' disease when given in conjunction with thiouracil—a two-action theory of iodine. J. Clin. Invest., 1945, 24, 869.
11. Goodman, L., and Gilman, A., The Pharmacological Basis of Therapeutics. New York, Macmillan, 1941.
12. Seymour, W. B., Jr., Poisoning from cutaneous application of iodine; rare aspect of its toxicologic properties. Arch. Int. Med., 1937, 59, 952.
13. Barker, W. H., and Wood, W. B., Severe febrile iodism during the treatment of hyperthyroidism. J. A. M. A., 1940, 114, 1029.
14. Finkelstein, R., and Jacobi, M., Fatal iodine poisoning; a clinico-pathologic and experimental study. Ann. Int. Med., 1937, 10, 1283.
15. Liebesny, P., Beitraege zur Pathologie des respiratorischen Gaswechsels. III. Mitteilung. Ueber den Einfluss des Jods auf den Stoffwechsel. Wien. klin. Wchnschr., 1924, 37, 521.
16. Veil, W. H., and Sturm, A., Beitraege zur Kenntnis des Jodstoffwechsels. Deutsches Arch. f. klin. Med., 1925, 147, 166.
17. Crotti, A., Thyroid and Thymus. Lea and Febiger, Phila., 1918.
18. Martin, K. A., Conditions under which iodine will cause change in basal metabolic rate in man; its occurrence in conditions other than that of Graves' disease. Am. J. M. Sc., 1927, 174, 648.
19. Snell, A. M., Ford, F., and Rowntree, L. G., Studies in basal metabolism. J. A. M. A., 1920, 75, 515.
20. Strouse, S., and Binswanger, H. F., Symptom complex resembling hyperthyroidism without increased metabolism; preliminary report. J. A. M. A., 1927, 88, 161.
21. Means, J. H., and Richardson, E. P., The Diagnosis and Treatment of Disease of the Thyroid. New York, Oxford University Press, 1938.
22. Kimball, O. P., Iodized salt for the prophylaxis of endemic goiter. J. A. M. A., 1946, 130, 80.
23. Webster, B., and Chesney, A. M., Endemic goitre in rabbits; effect of administration of iodine. Bull. Johns Hopkins Hosp., 1928, 43, 291.
24. Loeser, A., Die Umstimmung der Schilddrüsentätigkeit durch Jod. Klin. Wchnschr., 1934, 13, 533.
25. Siebert, W. J., and Linton, C. S., Comparison of effects of potassium iodide and of diiodotyrosin upon basal metabolism. J. Pharmacol. & Exper. Therap., 1935, 53, 418.
26. Siebert, W. J., Effect of KI upon basal metabolism and respiratory quotient in thyroidectomized guinea pigs. Proc. Soc. Exper. Biol. & Med., 1932, 29, 1146.

27. Rabinovitch, J., Effect of feeding potassium iodide on proliferative activity of thyroid gland in guinea pigs. Am. J. Path., 1928, 4, 601.
28. Marine, D., Deutch, M., and Cipra, A., Effect of large doses of iodine on heat production in rabbits. Proc. Soc. Exper. Biol. & Med., 1927, 24, 657.
29. Grabfield, G. P., and Prentiss, A. M., Further studies on effect of iodides on human nitrogen metabolism. J. Pharmacol. & Exper. Therap., 1925, 25, 411.
30. Nelson, N., Palmes, E. D., and Weymouth, P. P., Determination of iodine in biological materials. To be published.
31. Wallace, G. B., and Brodie, B. B., Distribution of administered iodide and thiocyanate in comparison with chloride, and their relation to body fluids. J. Pharmacol. & Exper. Therap., 1937, 61, 397.
32. Trevorrow, V., Studies on the nature of the iodine in blood. J. Biol. Chem., 1939, 137, 737.
33. Man, E. B., Smirnow, A. E., Gildea, E. F., and Peters, J. P., Serum iodine fractions in hyperthyroidism. J. Clin. Invest., 1942, 21, 773.
34. Cohn, B. N. E., Absorption of compound solution of iodine from gastrointestinal tract, with special reference to absorption of free iodine. Arch. Int. Med., 1932, 49, 950.
35. Bean, W. B., and Eichna, L. W., Performance in relation to environmental temperature. Federation Proc., 1943, 2, 144.
36. Eichna, L. W., Bean, W. B., Ashe, W. F., and Nelson, N., Performance in relation to environmental temperature. Bull. Johns Hopkins Hosp., 1945, 76, 25.

OBSERVATIONS ON MEN PERFORMING A STANDARD AMOUNT OF WORK IN LOW AMBIENT TEMPERATURES

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The activities of the various armies engaged in this last war have made evident the necessity for adequate information on the responses and reactions of man to the stresses of widely differing environmental conditions. During the past few years great emphasis has been placed upon investigations attempting to delineate the reactions of man to hot environments. Only a very limited number of studies were made in regard to cold weather despite the fact that greater divergences from normal (temperate zone) temperature conditions occur in sub-arctic and arctic areas.

Physiological investigations at these very low temperatures are hampered to some extent by the necessity for excessive amounts of clothing, which makes analysis of the role of the body temperature-regulating apparatus difficult. Even though investigators have been forced to consider only very practical matters, a certain amount of basic information has accrued,—primarily concerning the most adequate means of providing external heat or retaining body heat through the medium of protective clothing. In this report the responses of men working in environments as low as − 46.7° C. are presented. This complements earlier data (1) on men who sat quietly for long periods of time at similar ambient temperatures.

METHODS

All experiments were conducted from the months of August through December in the laboratory cold room described in a previous report (1). The environmental temperatures were maintained within ± 0.5° C. Air movement during test periods was practically zero. Exposure of subjects to the environments from − 3.3° to − 46.7° C. was made in a random manner. Two to four tests were made on each subject in the various environments except on subject JO., who was not available for ambient temperatures of − 16.1° and − 20.4° C. The physical characteristics of the five subjects are presented in Table I. The men were dressed in either of two clothing combinations,[3] arctic or herringbone twill uniforms.[3] In general only a single test was made on each subject during the day and that between 9 and 12 o'clock. The occasional 2- and 3-hour walking periods were conducted in the afternoon. The test procedure was as follows: each subject rested for 1 hour in a cool room (24° C., 50 per cent RH), donned a thermocouple harness, and was dressed in one of the uniforms. Rectal and skin temperatures were taken and the subject, entering the cold room, stepped onto the moving belt of the treadmill. At the completion of the hour work period, the man left the cold room and did not re-enter until another experiment on the following day.

TABLE I

Physical characteristics of subjects

Subject	Age	Height	Weight	Surface area
		cm.	*kgm.*	*M².*
Ve.	19	172	60.4	1.69
Jo.	20	169	73.5	1.83
Gl.	19	164	62.1	1.66
Ca.	21	159	53.4	1.53
Kl.	19	175	70.8	1.83

The treadmill in use had a walking surface provided by a roughened rubber belt sliding on a rigidly supported maple slipway. All tests were conducted at a speed of 3.0 miles per hour and a grade of 3.3 per cent. Skin temperatures were measured at 10-minute intervals at five or ten points with copper constantan thermocouples, and the mean skin temperature was calculated by the procedure suggested by Hardy and DuBois (2). Rectal temperatures were obtained either by calibrated clinical

[1] Major, MC, USA.

[2] T/3, MC, USA.

[3] *Arctic uniform:*

Drawers, wool, 50/50
Undershirt, wool, 50/50
Trousers, field, pile
Trousers, field, cotton, O.D. (sateen 9 oz.)
Parka, field, cotton, O.D. (sateen 9 oz.)
Parka, pile
Shoes, arctic, felt
Socks, wool, ski (2 pairs)
Socks, wool, cushion sole (1 pair)
Mittens, insert, trigger finger, M-1943
Mittens, shell, trigger finger, M-1943.

Herringbone twill uniform:

Suit, work, one piece herringbone twill
Shorts, cotton
Socks, wool, ski (1 pair)
Shoes, arctic, felt.

thermomters or by indwelling rectal thermocouples. Expired air was collected in compensated gasometers and analyses of aliquot gas samples collected over mercury were performed in duplicate using the Haldane machine. These machines were checked by daily analysis of outdoor air. Calculations for the oxygen consumption, respiratory quotient, and caloric expenditure were made in the usual manner. Inspired air was at the same temperature as the environmental. During the work periods, the respiratory exchange was determined after 10 and 45 minutes of walking. Collections of expired air were made for 10-minute periods. Heart rate by palpation was obtained on four occasions, before work and at 20, 40, and 55 minutes from the onset of walking.

RESULTS

The subjects were given preliminary training on the treadmill for a period of 12 days. Control studies were made at 24° C. on the 2 days prior to the first cold room exposure and again on the 2 days following the last low temperature day. No after-stimulating effect of cold was noted, the results being very similar to the pre-cold tests. Consequently all four tests at 24° C. were averaged to give the single control value in the tables. The data obtained on the men when dressed in the arctic uniform and working in the following eight low temperatures, − 3.3°, − 16.1°, − 20.4°, − 24.8°, − 27.2°, − 31.0°, − 37.2°, and − 46.7° C., are to be found in Tables II and III. Additional information is shown graphically in Figures 1 to 4.

Regardless of the type of clothing worn, the energy expenditure for the standard work load was always higher in the cold environments,—the average increase being approximately 12 per cent. The greater caloric expenditure was not due to

TABLE II

Metabolic observations on 5 men dressed in arctic clothing and walking on a treadmill at 3.0 M.P.H. and a 3.3 per cent grade during their first hour of exposure to the described environmental temperatures

Environmental temperature	Ventilation		Respiratory quotient		Oxygen consumption		Heat production					
	l. per min.	Δ *per cent*		Δ *per cent*	*l. per min.*	Δ *per cent*	*cal. per hr.*	Δ *per cent*	*cal. per M². per hr.*	Δ *per cent*	*cal. per kgm. per hr.*	Δ *per cent*
Basal* +24.0° C.	24.9		.85		1.18		345		207		5.7	
− 3.3° C.	27.3	9.6	.86	1.2	1.25	5.9	366	6.1	215	3.9	5.8	1.8
−16.1° C.	27.0	8.4	.90	5.9	1.31	11.0	387	12.2	218	5.3	5.8	1.8
−20.4° C.	26.8	7.6	.82	−3.5	1.33	12.7	384	11.3	221	6.8	5.9	3.5
−24.8° C.	26.4	6.0	.84	−1.2	1.33	12.7	388	12.5	226	9.2	6.1	7.0
−27.2° C.	28.1	12.8	.85	0.0	1.38	16.9	401	16.2	235	13.5	6.3	10.5
−31.0° C.	28.0	12.4	.86	1.2	1.31	11.0	384	11.3	222	7.2	5.9	3.5
−37.2° C.	27.1	8.8	.86	1.2	1.32	11.9	386	11.9	222	7.2	5.9	3.5
−46.7° C.	27.4	10.0	.86	1.2	1.32	11.9	387	12.2	226	9.2	6.1	7.0

* Wore herringbone twill uniform.

TABLE III

Temperatures (° C.) and heart rate of 5 subjects dressed in arctic clothing going into recorded temperatures and immediately starting to walk on the treadmill at 3.0 M.P.H. at a 3.3 per cent grade

Environmental temperature	Rectal temperature			Mean skin temperature			Toe temperature			Heart rate per min.	
	Before	After	Δ	Before	After	Δ	Before	After	Δ	Range	Average
Basal* +24.0° C.	37.3	37.9	0.6							85–110	103
− 3.3° C.	37.2	37.8	0.6	33.1	33.7	0.6	21.6	34.7	13.1	102–118	110
−16.1° C.	36.8	37.5	0.7	31.5	33.4	1.9	22.8	33.9	11.1	102–105	104
−20.4° C.	37.2	37.9	0.7	32.5	31.1	−1.4	26.9	32.6	5.7	81–102	95
−24.8° C.	37.3	38.1	0.8	33.5	30.0	−3.5	30.5	32.8	2.3	82–114	103
−27.2° C.	37.3	37.7	0.4	33.0	32.2	−0.8	26.7	34.4	7.7	88–110	102
−31.0° C.	37.1	37.7	0.6	33.0	31.2	−1.8	26.1	31.1	5.0	78–118	108
−37.2° C.	37.2	38.0	0.8	32.9	31.4	−1.5	28.8	33.3	4.5	72–126	106
−46.7° C.	37.2	38.2	1.0	33.2	29.4	−3.8	21.7	30.7	9.0	100–110	107

* Wore herringbone twill uniform.

the additional clothing worn, since the weight of the herringbone twill uniforms was the same at 24° C. as at the lower temperatures. For some inexplicable reason the highest values were always obtained at the envinronmental temperature of − 27.2° C. The smallest increase in caloric output was observed in the least cold environment, − 3.3° C.; in fact, when the herringbone twill uniform was worn, no differences from the control values were noted. However, in environmental temperatures above this, the increases were roughly similar for both garment assemblies. Cold appeared to provide a small but definite stimulus to the metabolic processes.

The average oxygen consumption of all the subjects at the different environmental temperatures is plotted in Figure 1, and, in Figure 2, a smiliar plot is presented for subject JO. These latter data, unfortunately not for every ambient temperature, are presented because his oxygen consumption at 24° C. was the same as the average for the group. Furthermore, JO, showed the greatest change of the five men at the ambient temperature of − 3.3° C.

The ventilation rate was also raised but in approximately the same proportion as the oxygen consumption. The frequency of breathing was not increased, the larger ventilation being compensated by adjustment in depth of respiration. Barcroft and Verzar (3) noted some irregular gasping in the breathing pattern of their subjects exposed to low temperatures, but no abnormalities were noted in this study, even at the lowest environmental temperature, − 46.7° C. The respiratory quotient exhibited no consistent change, being higher at some environments, lower at others, and the same as the control (24° C.) in the remainder.

The heart rate during work showed no significant changes with varying ambient temperatures, and the amount of clothing worn made no significant difference.

The changes in skin and rectal temperatures were quite different with the two types of clothing worn and will be discussed separately in the following sections.

Arctic clothing. This clothing combination has an insulative value between 4 and 5 Clo. and was

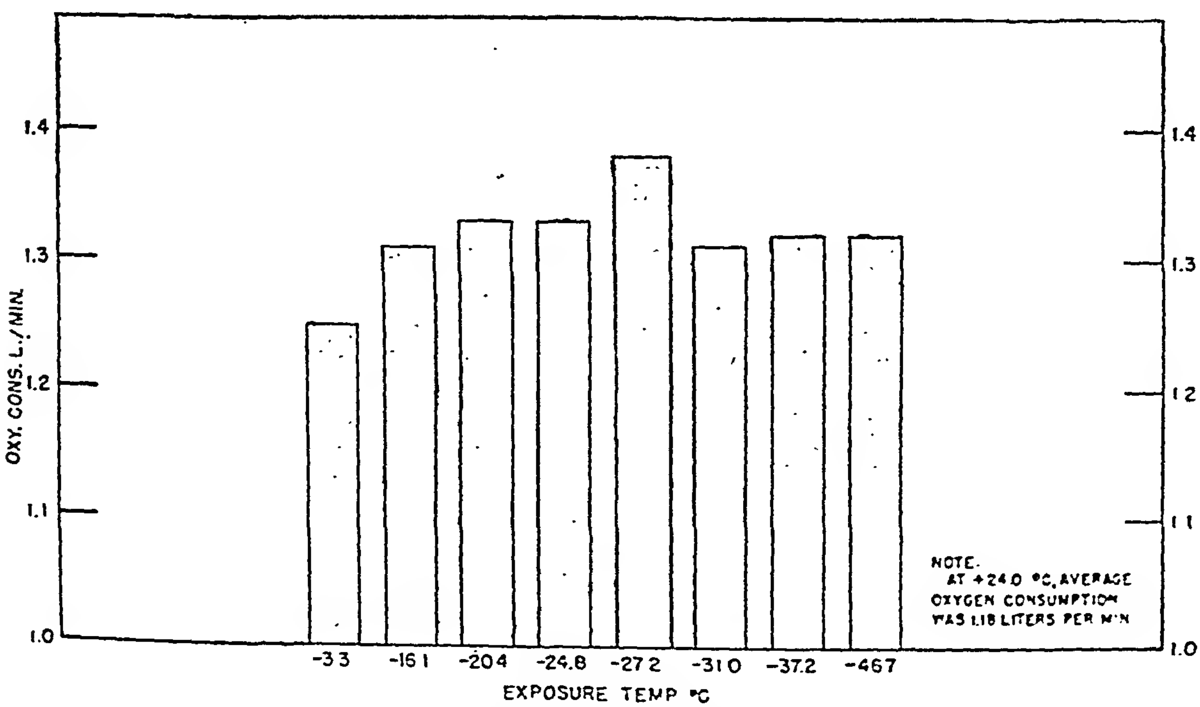

FIG. 1. OXYGEN CONSUMPTION OF FIVE (5) SUBJECTS DRESSED IN ARCTIC CLOTHING AND WALKING ON TREADMILL AT 3.0 M.P.H. AND A 3.3 PER CENT GRADE DURING THEIR FIRST HOUR OF EXPOSURE TO THE INDICATED TEMPERATURES

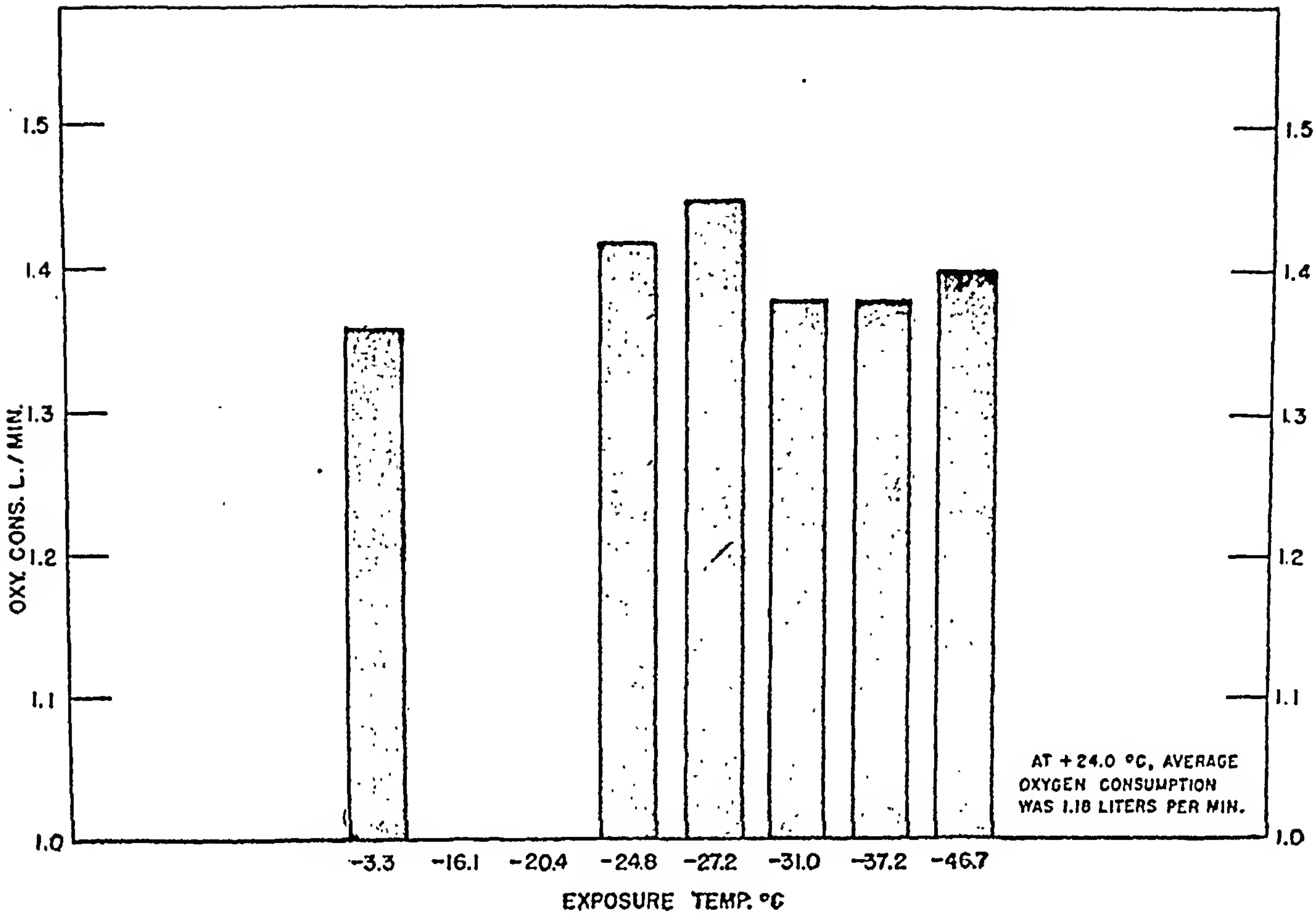

FIG. 2. OXYGEN CONSUMPTION OF ONE SUBJECT (JO.) DRESSED IN ARCTIC CLOTHING AND WALKING ON TREADMILL AT 3.0 M.P.H. AND A 3.3 PER CENT GRADE DURING HIS FIRST HOUR OF EXPOSURE TO THE INDICATED TEMPERATURES

worn completely buttoned during the walk. It was thought that the increased energy expenditure might be due to the weight of this outfit, 10 kilograms, but subsequent experiments with the herringbone twill issue and one experiment on a subject completely nude except for socks and shoes at − 20.4° C. indicated that the higher caloric output was primarily due to the stimulus of the low ambient temperature. Rectal temperatures were elevated to the same degree in normal and very cold environments. Only at − 46.7° C. was the increase significantly higher, 0.4° C. above the control, which was probably related to the greater vasoconstriction occurring at this low ambient temperature. Toe temperatures rose in all cases (Figure 3 and Table III), but the increases were somewhat erratic due to the variations in initial toe temperatures. Again the final level was approximately the same at all environments except at − 47.7° C.—a finding which bears out the suggestion of a more generalized vasoconstriction at this ambient temperature.

The explanation for finding higher final toe temperatures than mean skin temperatures is not available. It may have been due to a vasodilation being present in the extremities or to local friction effects. When subjects walk in hot environments, toe and foot temperatures as high as 3° to 5° C. above mean skin temperatures are frequently observed and could be explained as a consequence of friction.

The initial mean skin temperatures were quite constant. In the two least severe environments, final mean skin temperatures showed an actual increase, but beginning with − 20.4° C. decreases were always observed. The lowest value was found at the lowest ambient temperature. Storage values, calculated according to Burton's (4) formula, followed a pattern similar to the changes in mean skin temperatures primarily because the

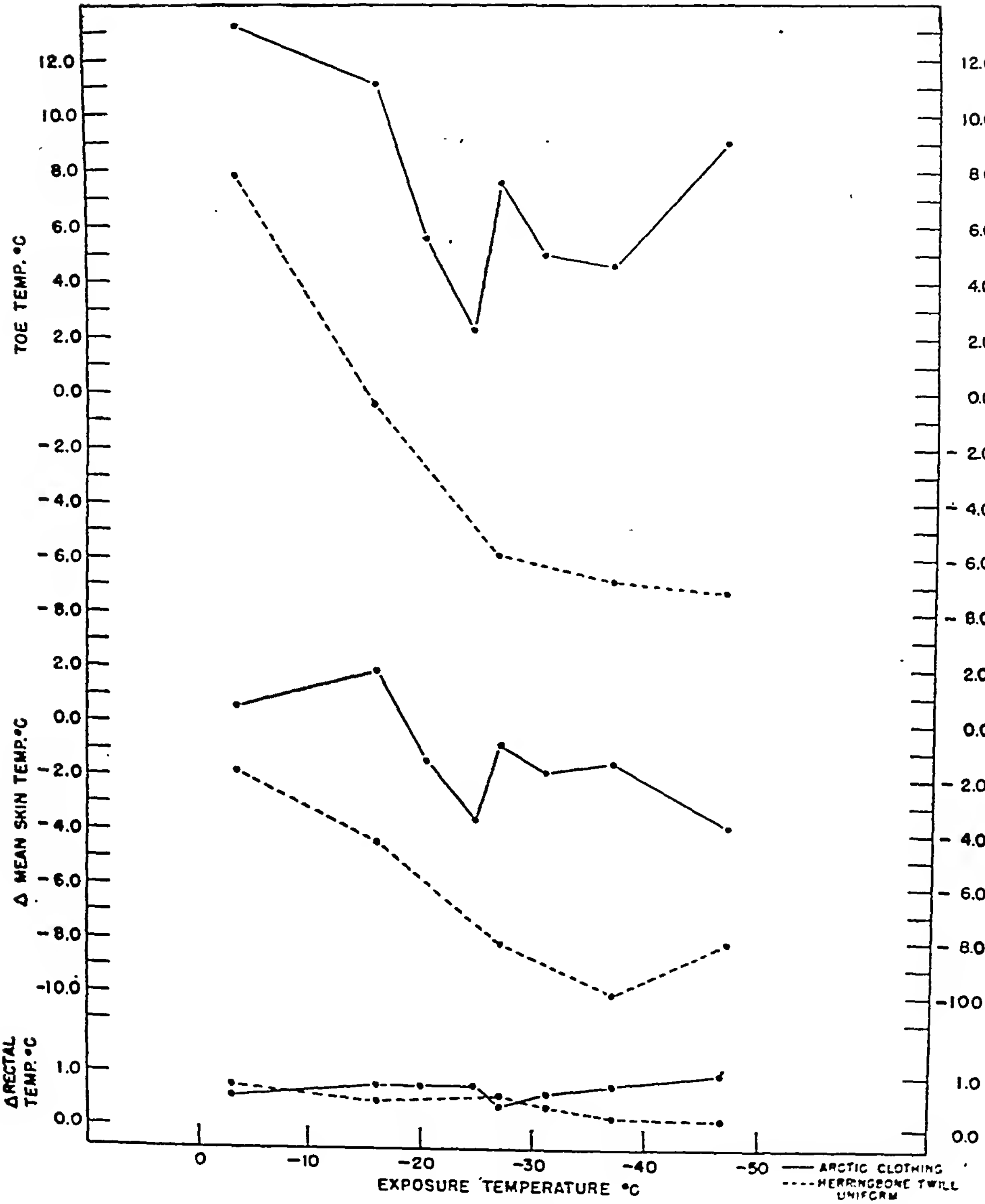

FIG. 3. INFLUENCE OF ENVIRONMENTAL TEMPERATURE AND CLOTHING ON THE MEAN CHANGES IN THE TEMPERATURE OF CERTAIN PARTS IMMEDIATELY BEFORE AND AFTER A 1-HOUR WALK ON THE TREADMILL AT 3.0 M.P.H. AT A 3.3 PER CENT GRADE

Data are the average of five subjects.

*Mean values for heat debts * incurred during work*

Uniform	Heat debt in cal. per M². per hr.							
	At −3.3° C.	At −16.1° C.	At −20.4° C.	At −24.8° C.	At −27.2° C.	At −31.0° C.	At −37.2° C.	At −46.7° C.
Arctic	−18.5	−34.1	0.0	19.9	0.0	6.2	−1.0	18.6
Herringbone twill	4.0	35.4	—	—	75.5	—	100.2	80.0

* A negative value indicates that heat was stored.

rectal temperature exhibited so little change from one environment to another.

Herringbone Twill. This clothing assembly weighs approximately 4 kilograms and has an insulative value between 1 and 2 Clo. There was no change in average calorie output at − 3.3° C. compared to the control environment of 24° C. (Table IV). However, as the environmental temperature decreased the energy expenditure increased, showing a maximum rise of 12 per cent at an ambient of − 46.7° C. The stimulative effect of cold was also reflected in the greater losses of body heat (see Table above). No appreciable debt was incurred at − 3.3° C., but at − 37.2° C., a debt of 100 cal. per M². was noted. There appeared to be a positive correlation between heat debt and ambient temperature at least up to − 37.2° C. These data on storage are reflections of the inability of the subjects to raise their rectal and mean skin temperatures despite the production of almost 400 calories per hour (Table V). The toe temperatures were increased only at − 3.3° C. At all other environments the toe temperatures fell despite the exercise (Figure 3), the final values being lower the colder the environment.

TABLE IV

Metabolic observations on 5 men dressed in herringbone twill coveralls walking on a treadmill at 3.0 M.P.H. and a 3.3 per cent grade during their first hour of exposure to the described environmental temperatures

Environmental temperature	Ventilation		Respiratory quotient		Oxygen consumption		Heat production					
	l. per min.	Δ *per cent*		Δ *per cent*	*l. per min.*	Δ *per cent*	*cal. per hr.*	Δ *per cent*	*cal. per M². per hr.*	Δ *per cent*	*cal. per kgm. per hr.*	Δ *per cent*
Basal +24.0° C.	24.9		.85		1.18		345		207		5.7	
− 3.3° C.	25.8	3.6	.88	3.0	1.18	0.0	346	0.3	202	−2.4	5.4	−5.3
−16.1° C.	26.9	8.0	.89	4.7	1.29	9.3	379	9.8	224	8.2	5.9	3.5
−27.2° C.	25.2	1.2	.83	−2.4	1.27	7.6	361	4.6	219	5.8	6.0	5.3
−37.2° C.	27.2	9.2	.87	2.4	1.29	9.3	377	9.3	223	7.7	6.0	5.3
−46.7° C.	26.8	7.6	.86	1.2	1.32	11.9	388	12.5	227	9.7	6.1	7.0

TABLE V

Temperatures (° C.) and heart rate of 5 subjects dressed in herringbone twill coveralls going into recorded temperatures walking on treadmill at 3.0 M.P.H. and at 3.3 per cent grade during their first hour of exposure to the described environmental temperatures

Environmental temperatures	Rectal temperature			Mean skin temperature			Toe temperature			Heart rate per min.	
	Before	After	Δ	Before	After	Δ	Before	After	Δ	Range	Average
° C.											
− 3.3	37.2	37.9	0.7	32.8	31.0	− 1.8	26.5	34.4	7.9	93–114	104
−16.1	37.6	38.1	0.5	33.6	29.2	− 4.4	32.8	32.5	−0.3	88–114	104
−27.2	37.5	38.1	0.6	34.0	25.9	− 8.1	33.6	27.7	−5.9	82–116	99
−37.2	37.6	37.9	0.3	34.0	24.0	−10.0	31.0	24.4	−6.6	102–116	109
−46.7	37.6	37.8	0.2	33.2	25.1	− 8.1	30.7	23.5	−7.2	88–116	104

DISCUSSION

There is no evidence that man is seriously handicapped in his ability to work in cold environments. As suggested by the increased energy expenditure for the same work output, the net efficiency for grade walking by these subjects was slightly reduced by the cold. However, additional work loads must be studied before a final statement regarding changes in efficiency of performance can be made. Adolph and Molnar (5) stated that "when the air was too cold, extra heat was produced above that which was a by-product of the work." The caloric output of their subjects at an environmental temperature of 37° to 39° F. was 299 cal. per $M.^2$ per hr. in contrast to 190 at an ambient temperature of 40° to 46° F. Such a degree of stimulation was not observed in our very lightly clothed subjects at environmental temperatures of − 46.7° C. (− 52.1° F.). Their subjects sometimes shivered while working—an observation never noted in this study. The subjects in this study never felt more than slightly chilly and in general were fairly comfortable.

In the group of experiments at − 20.4° and − 37.2° C., during which men walked for periods up to 3 hours, no additional changes were observed above those recorded for the 1-hour tests. Caloric expenditures appeared to decrease slightly, rectal temperatures rose approximately 0.1° C. per hour, and the mean skin temperatures were similar to those obtained at the end of the first hour. A balance in the heat exchange appeared to have been reached.

Heart rates were not significantly different at normally comfortable and extremely cold environments regardless of the amount of clothing worn. Previous reports (5) have suggested some correlation between the pulse rates of working men and lowered environmental temperatures. The differences between the results of this study and that of Adolph and Molnar (5) might be explained on the basis of clothing, since their subjects were nearly nude, but a disparity in clothing insulation of 2 Clo. would not be likely to compensate for a temperature differential of nearly 90° F. However, the effects of cold on sensory phenomena and cold tolerance may be greatly influenced by clothing and so affect the physiological measurements under consideration. Additional studies are clearly needed.

The major differences observed in the responses of the lightly and heavily clothed working subjects were in their rectal and skin temperatures. The rectal temperature rise in the cold was maintained at the same or even slightly higher level in working men wearing arctic suits as in a comfortable environment. Lightly dressed subjects had difficulty in reaching or maintaining this level except in the least cold ambient temperature. The colder the environment, the smaller was the elevation of the rectal temperature. The mean skin temperature exhibited a similar pattern in that large decreases were observed for the lightly clothed individuals with relatively small falls or actual increases for the heavily clothed.

In Figure 4, are plotted the data obtained on the mean skin and toe temperatures of a subject during one of these work periods at three of the lowest environmental temperatures employed. These changes in mean skin temperature with the wearing of the two clothing issues are typical. The major portion of the decrease occurred during the first 10 minutes of work and was maintained at a relatively constant level thereafter. Therefore, the major portion of the heat debt accumulated by the body occurred not only in a short period of time but also relatively early in the exposure. Measurements of the caloric expenditure of the subjects during these first 10 minutes indicated that the rate was the same then as during later portions of the work period. A similar type of response was noted in sitting subjects (1) where the greatest body heat loss also took place in the first phase of the exposure. This initial contribution appeared to be of vital importance in maintaining the working balance of heat exchanges in men exposed to low temperature. The significance of rapid loss of body heat in the regulation of body temperature was not evident from these studies.

The toe temperatures of the same subject (Figure 4) are interesting in that they not only showed the general pattern of response, but they reached the lowest level attained by any of the subjects during work. When the arctic uniform was being worn, an initial fall was noted which in almost every case then proceeded to rise, reaching stable

levels within a half hour after the beginning of the work period. This was not true when the lighter clothing assembly was worn, under which condition the toe temperature continued to go down after an initial fall. The rate of decrease diminished considerably as the experiment continued. The general beneficial effects of greater total insulation are obvious from these data.

The caloric expenditure for a given amount of work was slightly greater in the cold regardless of the degree of insulation provided by the clothing. The difference in the weights of the two

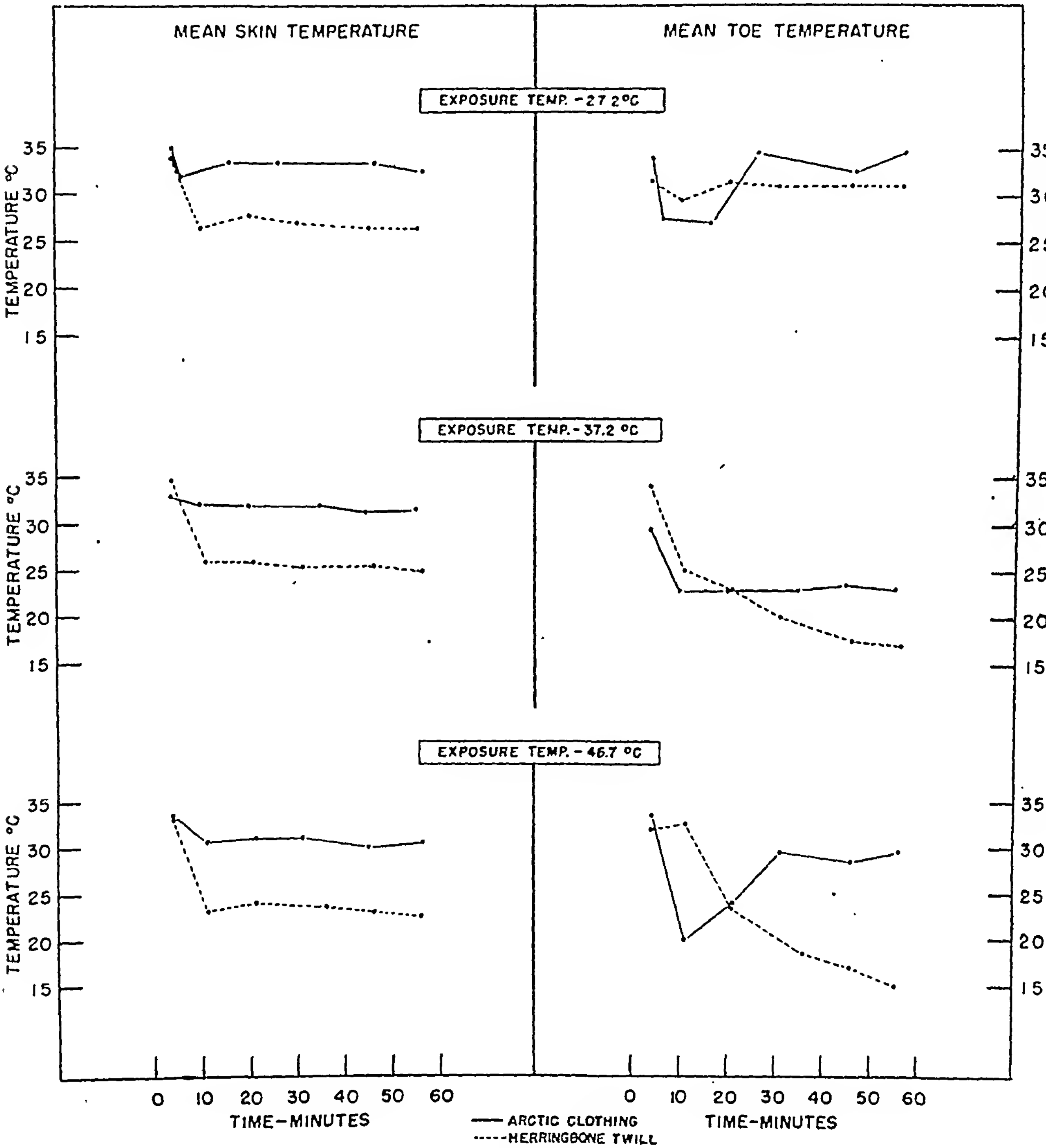

Fig. 4. Comparison of the Rate and Degree of Change of Mean Skin and Toe Temperatures in a Subject During a 1-Hour Walk Dressed in Either a Full Arctic Uniform or a Pair of Herringbone Twill Coveralls

garment assemblies resulted in only minor variations in the extent of the stimulation to metabolic processes. The diminution of mechanical efficiency in these subjects was of small magnitude. It might be explained as being due to the increased cost of respiratory effort, increased viscosity of cold muscles, or any number of small factors that inhibit the development of maximum skill and ease of work performance.

Men dressed in the arctic uniform can strike a balance of heat exchange while working at a caloric expenditure of around 350 calories per hour in ambient temperatures as low as − 46.7° C. However, this is only a partial answer to the adequacy of this type of clothing, since the accumulation of perspiration in the fabrics will alter a man's ability to maintain a balanced heat exchange, especially when he stops work. With very light dress, heat balances are not so readily attained at very low ambient temperatures. The degree of stress may be roughly estimated from the heat debt accrued. Caution in the use of these figures must be exercised, since the calculation of heat debt as presently performed may not be valid in cold environments.

No evidences of acclimatization to cold were found in this study. The intermittent nature of the exposures and the relatively short periods of time spent in the cold probably prevented the development of any such effect.

SUMMARY

1. Five subjects, wearing light and heavy clothing, walked at a standard rate of speed and grade of climb on a motor-driven treadmill in comfortable and very cold environments.

2. The energy expenditure for the standard work was increased by some 10 per cent in the lower ambient temperatures. This stimulation appeared to be independent of the amount of clothing worn. Ability to work was not significantly impaired even at ambient temperatures as low as −46.7° C., although there was a slight diminution of mechanical efficiency at certain of the environments.

3. The loss of body heat was greater with a light weight clothing and the magnitude of the loss appeared to be directly related to the ambient temperature. Heat debts were less but quite variable when heavy clothing was worn. In all cases, the heat debts were accumulated in the early portion of exposure to the cold environment.

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BIBLIOGRAPHY

1. Horvath, S. M., Golden, H., and Wagar, J., Some observations on men sitting quietly in extreme cold. J. Clin. Invest., 1946, 25, 709.
2. Hardy, J. D., and DuBois, E. F., The technic of measuring radiation and convection. J. Nutrition, 1938, 15, 461.
3. Barcroft, J., and Verzar, F., The effect of exposure to cold on the pulse rate and respiration of man. J. Physiol., 1931, 71, 373.
4. Burton, A. C., Human calorimetry. The average temperature of the tissues of the body. J. Nutrition, 1935, 9, 261.
5. Adolph, E. F., and Molnar, G. W., Exchanges of heat and tolerances to cold in men exposed to outdoor weather. Amer. J. Physiol., 1946, 146, 507.
6. Wolf, S., and Hardy, J. D., Studies on pain: Observations on pain due to local cooling and on factors involved in the "cold pressor" test. J. Clin. Invest., 1941, 20, 521.

CIRCULATORY DYNAMICS IN THE BASAL STATE OBSERVED DURING CONVALESCENCE. CHANGES IN BODY WEIGHT, BLOOD VOLUME, AND VENOUS PRESSURE [1, 2]

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Complaints of the circulatory symptoms of palpitation, tachycardia out of proportion to exertion, postural vertigo, and dyspnea are frequent among convalescents as a group. The present study was undertaken to determine if the commonly employed methods of evaluating circulation could be used in the prognostic classification of convalescents, and if such methods would indicate underlying physiological disturbances susceptible to modification with an ultimate shortening of the recovery period.

MATERIAL AND PROCEDURES

The 20 male subjects studied were civilians of military age who had had acute infectious diseases or surgical operations from which recovery was expected within a reasonable period of time. The purpose and general nature of the procedures were explained to the patients who volunteered to cooperate, although some were not too enthusiastic. Studies were begun within a few days of the subsidence of obvious manifestations of illness, such as tachycardia and fever, and at the time when clinical judgment indicated that convalescence had indeed begun. A number of these patients returned for follow up study upon regaining their usual good health. Fourteen of these follow up studies were satisfactory.

Difficulties were encountered in choosing and setting up a series of tests of circulatory function in convalescents. The earlier procedures, requiring several hours for completion, were too rigorous as evidenced by manifestations of severe malaise, nausea, vomiting, diarrhea, or headache. Patients complained bitterly upon their return to the ward and vowed not to participate further in the studies. However, as these individuals had no fever or clinical signs of toxic reaction to the chemicals employed in various techniques, the same materials were used in subsequent and less rigorous schedules of tests. When the procedures were too long or caused discomfort the patients became restless and uncooperative before the end of the period of observation.

Since such stress did not seem advisable in early convalescence, and the results were invalidated by it, the schedule was modified to levels of exertion well tolerated by the patients who then remained cooperative throughout the period of study. The earlier results, obtained when the patients reacted unfavorably during or following the series of tests, were discarded because we are skeptical of the value of such tests under any but basal conditions or those of accurately measured and reproducible stress. Changes in circulation resulting from the rigors of the earlier studies were not further investigated.

The series of observations finally adopted for use required about 1½ hours to complete and included ballistocardiographic records, pulse and respiratory rate, arterial blood pressure, oxygen consumption, blood volume, venous pressure, hematocrit, circulation time, weight, height, electrocardiogram, and a teleroentgengram of the chest.

The general nature of the procedure was explained to the patient, who cooperated purely upon a volunteer basis. Instructions were given to keep the patient in bed on the morning of the test, not to disturb him for routine care, and to delay the morning meal. He was brought to the laboratory upon a rolling stretcher, usually about 6:30 or 7:00 A.M. Here he was transferred to the ballistocardiograph bed (1) and allowed to rest undisturbed for ½ hour. The ballistocardiographic deflections were then photographed. Following this, the pulse and respiration rate were recorded and the arterial blood pressure determined with a mercurial manometer. Then, by using a Benedict-Roth closed breathing circuit, a respiratory tracing over a 7-minute period was obtained in duplicate.

The blood volume was estimated by the method of Gibson and Evans (2). Four samples of blood were delivered into Gibson tubes containing 20 volumes per cent of isotonic potassium oxalate for hematocrit values. Samples of serum were reserved for determination of total protein by the copper sulphate specific gravity method (3). During the blood volume determination, the venous pressure was measured directly with a saline manometer referred to a point 6 cm. below the angle of Louis with the patient supine (4). After completion of the blood volume determination, the blood velocity was

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Vanderbilt University.

[2] This work was done in the Lung Station of the Department of Medicine, a laboratory organized under a grant from the Commonwealth Fund, and some of the equipment used was available through a grant by the Ciba Pharmaceutical Products, Inc.

measured using Macasol [3] by the method of Spier, Wright, and Saylor (5). An electrocardiogram was taken. The patient was then weighed, measured, and transferred by roller stretcher to the X-ray room where a 6-foot film was obtained with the patient erect, either sitting or standing.

The ballistocardiograph employed in this study has been described elsewhere (6) and had physical properties essentially identical with the instrument of Starr and associates (1). The cardiac output was calculated by the area formula (1), modified slightly for the method of standardization employed by the authors. The cardiac output per minute per pound of body weight was compared with Starr's normal standard (7). Starr's normal data were recalculated in terms of cardiac output per calory of predicted basal metabolic rate, with slight improvement in deviations for sex and age, and our findings compared with their recalculated standards.

Heat production was calculated in the usual way from oxygen consumption and compared with predicted values from the Aub-DuBois standard (8). The difference was expressed as a percentage of normal as the usual basal metabolic rate.

Ventilation per minute was determined by integrating the respiratory tracing, and the utilization coefficient of Herbst (9) was derived from this and from the oxygen consumption, expressed as milliliters of oxygen consumed per liter ventilated.

Plasma volumes were calculated from curves of blue dye disappearance, and the total blood volume and cell volume were derived by the use of the hematocrit ratio from antecubital venous blood. Blood volumes were compared with the standards of Gibson and Evans (10) basing the prediction upon the height of the patient.

The transverse diameter of the heart was measured in the teleroentgengram as was the internal diameter of the chest at the level of the fourth costochondral junction. In our experience this latter measure is less subject to irrelevant variation than the usual measure made at a lower level in the rib skirt. The ratio of the heart diameter to the internal diameter of the chest was then calculated.

TABLE I

Mean values of observations of convalescent male civilians of military age and the mean difference upon recovery

	No. of cases	Initial mean and standard deviation	No. of cases	Mean difference upon recovery
Weight, kgm.	20	62.25 ± 9.80	14	+ 3.60*
Respiratory rate per min.	19	16.50 ± 2.80	13	− 1.80
Blood pressure, mm. Hg				
Systolic	20	112.80 ± 7.80	14	+ 5.80
Diastolic	20	70.40 ± 9.90	14	+ 0.60
Av. systolic and diastolic	20	91.60 ± 9.30	14	+ 3.20
Venous pressure, mm. saline	20	71.60 ± 26.10	14	+24.60*
Circulation time, sec.				
Arm to tongue	20	19.10 ± 4.20	12	− 0.40
Arm to hand	19	27.90 ± 7.10	12	− 0.30
Arm to perineum	19	30.10 ± 9.20	10	− 0.70
Arm to foot	11	39.50 ± 11.70		
Cardiac output				
Per stroke, ml.	17	48.60 ± 11.30	9	− 0.80
Per min., liters	17	3.41 ± 0.57	9	− 0.29
Per lb. per min., ml.	17	24.20 ± 4.40	9	− 3.50
Per min. per cal. predicted B.M.R., ml.	17	47.60 ± 8.40	9	− 5.10
Per min. per cal. observed B.M.R., ml.	13	48.40 ± 7.10	7	− 0.20
B.M.R., per cent	17	−2.90 ± 9.50	10	− 3.10
Utilization coefficient	17	39.30 ± 7.60	9	+ 0.30
Ventilation, liters	17	5.90 ± 0.90	9	+ 0.10
Tidal air, ml.	17	443.00 ± 123.00	9	+21.20
Heart size, transverse diameter, cm.	19	12.40 ± 1.30	12	+ 0.40
Hematocrit value, vol. per cent	20	43.60 ± 5.60	14	+ 1.70
Blood volume, liters	20	5.35 ± 0.63	13	+ 0.46*
Plasma volume, liters	20	2.98 ± 0.32	13	+ 0.19*
Cell volume, liters	20	2.37 ± 0.49	13	+ 0.28*
Plasma total protein, grams per cent	19	6.70 ± 0.60	12	+ 0.20
Circulating plasma total protein	19	199.10 ± 26.00	12	+10.20

* Changes considered significant.

RESULTS

Data from various tests are given in Table I, including the mean values and standard deviations of the observations made during early convalescence, and the mean differences between these initial values and those following recovery. The mean values of the initial studies do not deviate from commonly accepted normal standards, with the exception of blood velocity. Whether this latter difference is real is discussed below.

There was no uniformity in the changes in systolic and diastolic blood pressure or in the pulse pressure recorded in early convalescence and recovery, since about half of the group showed an increase, the remainder a decrease in pressure.

From Table I, it is apparent that the blood velocity was the same in convalescence and health. The data from arm-to-foot determinations following recovery were too few to be included.

The values for the mean cardiac output per minute per pound of body weight in convalescence compare favorably with Starr's (7) figure of 23 ml. per minute per pound as the normal mean. The mean decrease of 3.5 ml. per minute per pound after recovery was due to the increase in body weight rather than to the small decrease in minute volume.

A correlation of the cardiac output per minute with the predicted and observed basal metabolic rate in the recovery and convalescent states was fruitless. Recalculation of Starr's data for normal subjects showed the mean normal value for cardiac output per minute per calory of the predicted B.M.R. to be 50 ml., with a range of 40 to 60 ml.,

[3] Supplied through the courtesy of the Nepera Chemical Co., Yonkers, New York.

corresponding to Starr's range of 18–29 ml. per minute per pound of body weight.

From the data obtained in these studies it cannot be said that the basal metabolic rate is useful as a criterion for convalescence. In central Tennessee, where all of these studies were made, general experience indicates that a mean basal value of minus 5 per cent by the Aub-DuBois standard may be regarded as normal; farther south, in New Orleans, the figure is minus 10 per cent (11).

As might be inferred from the data on ventilation and the basal metabolic rate, the relation between ventilation and the oxygen consumption expressed by Herbst's coefficient (9) was neither abnormal initially nor was it significantly altered subsequently. The mean coefficient was 39.3 ±

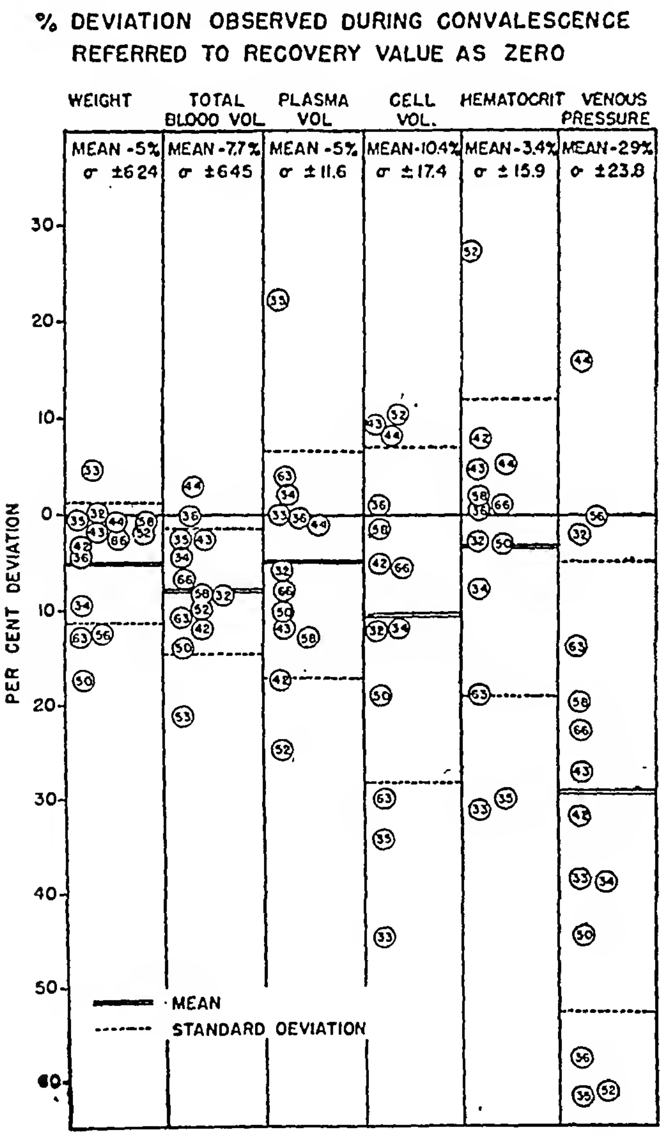

FIGURE 1.

TABLE II

Blood volume studies, blue dye method, of adult males

	No. of cases	Initial observations: mean	Mean difference 1st and 2nd obs.
House officers, normal males (13)	5	5.57 liters	+0.006 liter
Convalescents	20	5.35 liters	+0.461 liter*
Gibson's normal males (10)	49	5.31 liters	

* This represents the mean increase after recovery in the case available for a second observation.

The data on the 5 house officers studied on 2 occasions months apart are included to show that there is little deviation attributable to the method in the study of a group. The mean difference of 0.461 liter between the blood volumes observed in convalescence and in the same cases after recovery is contrasted to the lack of difference in the 5 normal males restudied.

7.6 ml. of oxygen consumed per liter ventilated. After recovery there was a mean increase of 0.31 ml. The normal mean obtained by Hurtado and Boller (12) who used the same procedure was 37.5 ml. oxygen per liter ventilated, with a coefficient of variation of ± 27 per cent. The mean increase of 0.37 cm. in the transverse diameter of the heart cannot be regarded as significant.

From the data it was obvious that weight differences between a patient's reported normal and his observed weight in early convalescence could not be relied upon as a criterion of convalescence. This is illustrated by a discrepancy between the weight loss reported and the weight regained by the individuals followed to recovery. While the patients appeared to have lost an average of 6.11 kgm., to judge from the difference between the patient's estimate of his usual weight and his actual weight during convalescence, yet upon return to usual health, the weight regained averaged only 3.59 kgm. (Table I and Figure 1).

Table II contains data on blood volume obtained from: (a) 5 house officers among whom there was no reason to consider a physical or medical change in status during the 6-month interval elapsing between the 2 determinations (13), (b) our convalescent and recovered groups or subjects, and (c) Gibson's mean of blood volumes in 49 normal males (10). The blue dye method of Gibson (2) was used in all of these studies. Many of the normal values included in Gibson's study were obtained from hospital-bed patients, which might explain the difference between his average total blood volume in males, 5.35 liters, and the aver-

age blood volume, 5.76 liters, in our group of recovered ambulatory subjects.

The increase in total volume of the blood was due to both plasma and cell increases, the latter change predominating in patients frankly anemic in the early convalescent state, the former in those with high hematocrit values in convalescence (Table I and Figure 1).

The venous pressure was significantly increased after recovery in most subjects; it was unchanged in one, and decreased 8 mm. saline in another, the mean increase being 24.6 mm. saline, the greatest, 73 mm. saline, representing a mean increase of 35 per cent (Figure 1 and Table I).

The electrocardiograms were within normal limits in convalescents and following recovery there was no significant change. Plasma total protein and circulating plasma total protein showed no significant change.

DISCUSSION

It is at once evident that the circulation at rest during early convalescence deviates only slightly from normal. The 2 particulars in which deviations were found were blood volume and venous pressure. To these might be added body weight, for some part of the weight deficit appears to be interstitial water (14).

The deficit in blood volume is of the same order of magnitude as that found by Keys and his associates (15) and by Barr and his associates (16) in normal men at bed rest, and by Rutstein (14) in convalescence from pneumonia. Loss of about ½ kgm. of body weight and about 100 grams of protein may be accounted for by the observed deficit in blood volume.

The question arises as to whether these blood volume changes are real. Cruickshank's (17) reiteration of Smith's (18) observation that some unknown fraction of the administered dye is taken up by the reticulo-endothelial system immediately is a valid criticism of the method. The discrepancy between venous and general body hematocrit values (19) is another. Although there is no evidence that errors in the blood volume are consistent on different occasions when the patient is in different clinical states, yet it is to be hoped this is the case so that changes observed in a patient from time to time may be real even though the absolute values are incorrect. The reproducibility of results obtained by the method of Gibson and Evans (2) may be seen in the normal house officers (Table II) who were studied at different times of the year.

The rise in venous pressure upon recovery is not explained. It was not related to changes in cardiac output, to blood volume, nor to any other measurement we made.

It is interesting to compare our findings with those of Lyons and his associates (20) who have recently reported extremely interesting observations on normal individuals who underwent diuresis with ammonium chloride and mercupurin. There was a loss in body weight of a little over 3½ kgm. associated with a fall in plasma volume slightly over 700 ml. Much of the diminution in body weight was aparently due to diminution of interstitial water. His subjects exhibited symptoms "suggestive of an inadequate circulation . . . they complained of weakness, prostration, fatigue, and some felt so faint attempting to stand that they chose to remain in bed." The striking similarity of these symptoms to symptoms experienced by patients during early convalescence is obvious, and possibly the mechanism of production of these manifestations is closely related in the 2 cases.

The values obtained for circulation time seemed long to us. However, as patients returned for follow up without change, we were compelled to reinvestigate the normal standards for this method in our hands. Forty-six normal men in the basal state formed the material for this review and the results are to be published elsewhere (21). Suffice it to say there was no significant difference between the blood velocity in these normals and that found in our early convalescents.

SUMMARY

1. The following measurements were made upon a group of basal convalescent male patients who had undergone infectious disease or surgical operation: height, weight, pulse, respiration, arterial and venous blood pressure, blood velocity from arm to tongue, hand, perineum and feet, cardiac output, cardio-thoracic ratio, oxygen consumption, basal metabolic rate, ventilation, tidal air, utilization coefficient, hematocrit value, total blood volume, plasma volume and cell volume, plasma

total protein, circulating plasma total protein, and an electrocardiogram. None of these observations indicated any characteristic abnormality of the circulation when the results were compared with currently accepted normal standards; so none of these tests is of any use as a routine procedure for the clinical classification of convalescence.

2. Follow up studies revealed small but significant alterations which could be detected only by comparing the individual with himself as a control. These differences were: a deficit in body weight of 3.6 kgm., a deficit during early convalescence of approximately ½ liter of blood volume, and a deficit of 35 per cent of the patient's usual venous pressure.

BIBLIOGRAPHY

1. Starr, I., Rawson, A. J., Schroeder, H. A., and Joseph, N. R., Studies on the estimation of cardiac output in man, and of abnormalities in cardiac function, from the heart's recoil and the blood's impacts; the ballistocardiogram. Am. J. Physiol., 1939, 127, 1.
2. Gibson, J. G., II, and Evans, W. A., Jr., Clinical studies of the blood volume. I. Clinical application of a method employing the azo dye "Evans blue" and the spectrophotometer. J. Clin. Invest., 1937, 16, 301.
3. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M., Copper sulfate method for measuring specific gravities of whole blood and plasma. The United States Navy Research Unit at the hospital of the Rockefeller Institute for Medical Research.
4. Griffith, G. C., Chamberlain, C. T., and Kitchell, J. R., A simplified apparatus for direct venous pressure determination modified from Maritz and V. Tabora. Am. J. M. Sc., 1934, 187, 371.
5. Spier, L. C., Wright, I. S., and Saylor, L., New method for determining circulation time throughout vascular system. Am. Heart J., 1936, 12, 511.
6. Meneely, George R., to be published.
7. Starr, I., and Schroeder, H. A., Ballistocardiogram. II. Normal standards, abnormalities commonly found in diseases of the heart and circulation, and their significance. J. Clin. Invest., 1940, 19, 437.
8. DuBois, E. F., Basal Metabolism in Health and Disease. Philadelphia, Lea and Febiger, 1927.
9. Herbst, R., Der Gasstoffwechsel als Mass der Körperlichen Leistungsfähigkeit. I. Die Bestimmung des Sauerstoffaufnahmevermögens beim Gesunden. Deutsches Arch. F. klin. Med., 1928, 162, 33.
10. Gibson, J. G., II, and Evans, W. A. Jr., Clinical studies of the blood volume. II. The relation of plasma and total blood volume to venous pressure, blood velocity rate; physical measurements, age, and sex in ninety normal humans. *J. Clin. Invest.*, *1937*, *16*, 317.
11. Andes, J. E., and Eaton, A. G., Synopsis of Applied Pathological Chemistry. 376. St. Louis, C. V. Mosby Company, 1941.
12. Hurtado, A., and Boller, C., Studies of total pulmonary capacity and its subdivisions. I. Normal absolute and relative values. J. Clin. Invest., 1933, 12, 793.
13. Kaltreider, N. L., Meneely, G. R., Allen, J. R., and Bale, W. F., Determination of the volume of the extracellular fluid of the body with radioactive sodium.. J. Exper. Med., 1941, 74, 569.
14. Rutstein, D. D., Thomson, K. J., Tolmach, D. M., Walker, W. H., and Floody, R. J., Plasma volume and "extra vascular thiocyanate space" in pneumococcus pneumonia. J. Clin. Invest., 1945, 24, 11.
15. Taylor, H. L., Erickson, L., Henschel, A., and Keys, A., The effect of bed rest on the blood volume of normal young men. Am. J. Physiol., 1945, 144, 227.
16. Barr, D. P., Dietrick, J., and Whedon, G. D., The effects of bed rest and immobilization on various physiological and chemical functions of normal man. The Conference on Metabolic Aspects of Convalescence, Including Bone and Wound Healing, Conference Reports of the Josiah Macy, Jr. Foundation, Feb., 1945.
17. Cruickshank, E. W. H., and Whitfield, I. C., The behaviour of T. 1824 (Evans blue) in circulating blood and a modified method for the estimation of plasma volume. J. Physiol., 1945, 104, 52.
18. Smith, H. P., The fate of an intravenously injected dye (brilliant vital red) with special reference to its use in blood volume determination. Bull. Johns Hopkins Hosp., 1925, 36, 325.
19. Hahn, P. F., Ross, J. F., Bale, W. F., Balfour, W. M., and Whipple, G. H., Red cell and plasma volumes (circulating and total) as determined by radio iron and by dye. J. Exper. Med., 1942, 75, 221.
20. Lyons, Richard H., Jacobson, Samuel D., and Avery, Noyes L., The change in plasma volume and body weight in normal subjects after a low salt diet, ammonium chloride and mercupurin. Am. J. M. Sc., 1946, 211, 460.
21. Meneely, G. R., and Segaloff, Albert. To be published.

SULFADIAZINE RESISTANT STREPTOCOCCAL INFECTIONS IN A CIVILIAN COMMUNITY [1, 2]

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During a program of mass chemoprophylaxis of streptococcal infections in the Armed Forces (1), it was noted that the effectiveness of such a regimen was lost with the appearance of sulfonamide-resistant strains of hemolytic streptococci. The occurrence and spread of these resistant strains in susceptible populations in the U. S. Army and Navy have been reported (2, 3, 4), and have been the subject of considerable speculation and theoretical implications. To date there have been no reports as to the incidence of streptococcal epidemics in the civilian population due to sulfonamide-resistant strains. It is the purpose of this paper to report the occurrence of such an epidemic in a civilian community due to a sulfadiazine-resistant group A, type 19 hemolytic streptococcus.

METHODS OF STUDY

Early in 1946 a small epidemic of scarlet fever occurred in Cooperstown, New York. Since the first 8 patients with scarlet fever were found to harbor type 19 hemolytic streptococci in their nasopharynges, it was decided to determine whether we were dealing with sulfonamide-resistant strains. All 8 strains of hemolytic streptococci were found to be resistant to the action of sodium sulfadiazine in a concentration of 25 mgm. per cent.

Because of the unique opportunity to study the incidence of sulfonamide-resistant hemolytic streptococci in a small community, it was decided to test the sulfonamide sensitivity of all hemolytic streptococcus cultures sent to the laboratory in this area. Cultures were obtained from patients, living in Cooperstown, New York, and the surrounding area of Otsego County, who were suspected of having streptococcal infections. Records were kept as to the source of the cultures and the clinical diagnosis. No attempt was made to do a widespread survey on a healthy group to determine carrier rates.

All culture swabs were sent to the Otsego County Laboratory at the Mary Imogene Bassett Hospital or to a nearby branch laboratory. The swabs were streaked on 5 per cent rabbit blood agar which was incubated for 24 hours at 37° C. Single colonies of beta hemolytic streptococci were transferred to Avery's media on the following day and incubated for 6 hours. A loopful of this growth was then streaked to a blood agar plate, checked for purity, and the group determined. The group A hemolytic streptococci isolated were grouped and typed at the Rockefeller Institute Hospital by the precipitin technique (5), and the sulfonamide sensitivity was determined by the method of Wilson (6).

EPIDEMIOLOGICAL SITUATION AND RESULTS

From February 15 to May 1, 1946, streptococcal infections were moderately prevalent in Cooperstown, New York. During this period 100 patients with varied group A hemolytic streptococcal in-

TABLE I

Type distribution and sulfadiazine resistance of group A hemolytic streptococci

Infecting type	No. of cases	No. of sulfadiazine-resistant strains
1	1	0
2	2	
3	28	
4	1	
5	2	
6	2	
9	1	
12	2	
13	1	
14	2	
17	1	
28	4	
NC[1]	29	
19	24	23[2]

[1] NC indicates strains not classified by the precipitin method with available diagnostic sera.

[2] These 23 strains all resisted 25 mgm. per cent of sodium sulfadiazine in the media.

[1] Assisted in part by a contract between The Rockefeller Institute for Medical Research and the Commission on Hemolytic Streptococcal Infections, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

[2] This article has been released for publication by the Division of Publications of the Bureau of Medicine and Surgery of the U. S. Navy. The opinions and views set forth in this article are those of the writers and are not to be considered as reflecting the policies of the Navy Department.

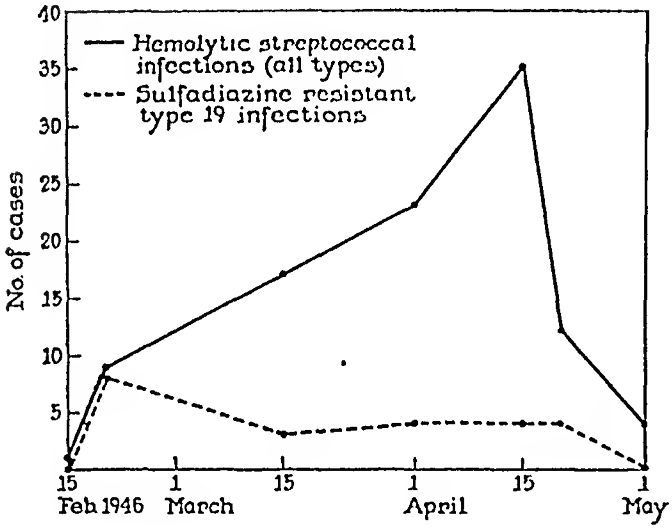

FIG. 1

fections were cultured, with the type distribution indicated in Table I. The cultures were obtained from patients with scarlet fever, pharyngitis, otitis media, wound infections, blood stream infections, and pneumonia. The time incidence of cases during this period is shown in Chart 1. By May 1, 1946, the incidence of streptococcal infections had decreased to the point where it was no longer considered of value to continue the study.

The most common typable streptococcus was type 3, which was responsible for 28 cases of hemolytic streptococcal infections. Type 19 was the cause of 24 cases of disease, with 23 of these strains being resistant to 25 mgm. per cent sodium sulfadiazine and 1 strain non-resistant. Nineteen cases were due to the other types listed in Table I, while 29 were caused by untypable strains. Only type 19 hemolytic streptococci were resistant to the action of sodium sulfadiazine.

Since our main interest in this report is concerned with the occurrence of type 19 sulfonamide-resistant hemolytic streptococci, the epidemiological situation in respect to these infections will be discussed briefly. Of the 23 persons infected with this sulfonamide resistant strain, 14 were under 15 years of age, 10 of public school age, and 4 of pre-school age, 9 cases occurred in adults.

The first case of scarlet fever in this study developed on February 15, 1946, in a 9-year-old boy who attended the public school. Following the onset of this patient's illness, several classmates developed scarlet fever, and from their nasopharynges type 19 sulfadiazine-resistant streptococci were isolated. The next cases developing due to resistant type 19 strains appeared in parents, sibblings, or contacts of the children originally infected in the public school. However, 3 patients were from widely separated communities and had no known contact with any of the previously mentioned cases. None had any known contact with recently discharged or active members of the Armed Forces.

Of these cases of drug-resistant infection, 14 were clinical scarlet fever, and 9 were merely pharyngitis. One case of scarlet fever and one case of pharyngitis were complicated by otitis media and one patient developed rheumatic fever following pharyngitis due to this type 19 sulfadiazine-resistant hemolytic streptococcus. None of these cases presented any special therapeutic problems, nor were they clinically different from infections due to other types of hemolytic streptococci. The incidence of infection with this particular organism was rather evenly distributed throughout the period of this study with the peak occurring early (see Figure 1).

COMMENT

The possibility of a spread of sulfadiazine-resistant hemolytic streptococci to the civilian population has been anticipated ever since their appearance in the Armed Forces (2, 3, 4). The present report indicates that such resistant strains have, indeed, been responsible for infections in at least one civilian community and that similar occurrences may complicate future problems of therapy among civilians.

The mode of development of resistant strains of bacteria is still a matter of discussion. It has been demonstrated that strains of streptococci with low or medium degrees of sulfadiazine resistance appeared in the Army (7) about the time that the program of mass sulfa-prophylaxis had been in progress in the Navy (1) for several months. It had been suggested that these slightly resistant strains were mutants that might possibly have been precursors of the more highly resistant strains later established. The possibility cannot, however, be eliminated that there was a transfer of resistant strains to soldiers from certain Naval personnel, who were subjects of the mass prophylaxis program, and hence possible carriers of highly resistant streptococci. It is noteworthy that no evidence of sulfonamide resistance was

found in any strains of hemolytic streptococci prior to the institution of the mass chemoprophylaxis programs (2, 8).

The possibility that sulfonamide-resistant hemolytic streptococci could arise in the civilian population must be considered. The evidence to date, however, would seem to indicate that all strains of hemolytic streptococci which were present in the civilian population prior to the time of mass chemoprophylaxis in the Armed Forces were susceptible to the action of the sulfonamides. Evidence (8) has also been presented to show that the usual therapeutic doses of sulfonamides, such as might be prescribed in a civilian population, did not cause the development of resistant organisms.

Since the sulfadiazine-resistant hemolytic streptococcal infections reported in this study were caused by a type 19 strain, which was known to be a prominent resistant strain in the Armed Forces, it seems probable that this strain was introduced into this civilian community by military personnel even though a history of recent contact with sick soldiers and sailors could not be elicited. Contact of civilians with discharged veterans, some of whom might have been healthy carriers of these resistant strains, could hardly have been avoided. The fact that most cases of sulfonamide-resistant infections here reported occurred among school children is noteworthy, since all previous reports of sulfonamide-resistant infections occurred in adults.

Although the cases mentioned in this report did not offer any problems as to therapy, one cannot overlook the importance of determining whether one is dealing with an organism sensitive to the chemotherapeutic agent being administered. Since the clinical picture of infections caused by resistant strains of hemolytic streptococci is no different than those caused by non-resistant strains, one can only determine this property by isolation and testing of the organism causing the infection. From an epidemiological and clinical point of view, the determination of the presence of resistant strains would be of definite value if the prevention and treatment of resistant-strain complications is to be effectively accomplished.

The appearance of increasing numbers of bacteria which are resistant to various chemotherapeutic agents, formerly effective against them, suggests the desirability of a careful bacteriological diagnosis of infectious agents prior to treatment with specific antibacterial drugs, especially when there is an obvious failure of clinical response to the chemotherapeutic agent under consideration.

SUMMARY

Twenty-three cases of type 19 sulfadiazine-resistant hemolytic streptococcus infections occurred in a civilian community. None of the cases presented any problem as to treatment nor were there any serious complications. Attention is directed to the importance of determining the sensitivity of infecting bacteria to chemotherapeutic agents prior to the start of treatment with specific agents.

The authors wish to thank Dr. Ann Kuttner and Dr. Homer F. Swift for their interest and criticism during this study.

Dr. Walter Eells, Walton, New York, and Dr. George Rosenthal, Cherry Valley, New York, were instrumental in obtaining many of the streptococcal cultures used in this study. Valuable technical assistance was rendered by Mrs. Winifred Cary, Cooperstown, New York, and Miss Jeanne Epstein of The Rockefeller Institute Hospital, New York, New York.

BIBLIOGRAPHY

1. Coburn, A. F., Mass sulfadiazine prophylaxis of respiratory diseases in the U. S. Navy. Bull. New York Acad. Med., 1945, 21, 281.
2. Sulfadiazine resistant strains of beta hemolytic streptococci: Appearance during the course of sulfadiazine prophylaxis at a large naval training center, Epidemiology Unit Number 22. J. A. M. A., 1945, 129, 921.
3. Damrosch, D. S., Chemoprophylaxis and sulfonamide resistant streptococci. J. A. M. A., 1946, 130, 124.
4. (a) Delameter, E. D., Jennings, R., and Wallace, A. W., Preliminary report of an outbreak of streptococcal disease caused by a sulfadiazine resistant group A, type 17 hemolytic streptococcus. J. Infect. Dis., 1946, 78, 118.
   (b) Mitchell, R. B., et al., The interpost dissemination of epidemic strains of hemolytic streptococci by troop movements. J. Infect. Dis., 1946, 78, 126.
   (c) Roberg, N. B., An epidemic caused by a sulfadiazine resistant strain of the streptococcus hemolyticus (group A, type 17). J. Infect. Dis., 1946, 78, 135.
   (d) Wilson, O. G., An outbreak of sulfadiazine resistant streptococcus infection at Lowry Field, Colorado. J. Infect. Dis., 1946, 78, 147.

5. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exper. Med., 1943, 78, 127.

6. Wilson, A. T., Method for testing in vitro resistance of group A hemolytic streptococci to sulfonamides. Proc. Soc. Exper. Biol. & Med., 1945, 58, 130.

7. Rantz, L. A., Randall, E., Spink, W. W., and Boisvert, P. J., Sulfonamide and penicillin resistance of group A hemolytic streptococci. Proc. Soc. Exper. Biol. & Med., 1946, 62, 54.

8. Hartman, T. L., Sulfonamide sensitivity determinations of hemolytic streptococci isolated from patients before and after treatment with sulfadiazine. Bull. Johns Hopkins Hosp., 1946, 79, 342.

STUDIES ON THE RELATIONSHIP OF THE HEPATITIS VIRUS TO PERSISTENT SYMPTOMS, DISABILITY, AND HEPATIC DISTURBANCE ("CHRONIC HEPATITIS SYNDROME") FOLLOWING ACUTE INFECTIOUS HEPATITIS [1]

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The term "chronic hepatitis" has been suggested by Barker, Capps, and Allen (1) for reference to those patients with infectious hepatitis who fail to recover within 4 months of the onset of the disease. This term also appears to be applicable to those patients with homologous serum hepatitis who fail to recover within 4 months. As used here the word "chronic" refers only to "duration without implication concerning the nature of the pathologic process or the eventual prognosis."

The frequency with which "virus hepatitis"[3] becomes chronic is not known and probably varies with the patient's age, general condition (nutritional and otherwise), management, and possibly with the strain of virus concerned. However, in a series of 431 unselected cases diagnosed as "infectious hepatitis" in the Mediterranean Theater (1), 18 per cent were classified as chronic on the basis of the above definition. Furthermore, under the favorable conditions associated with the study of induced infectious hepatitis in one group of healthy, well-nourished, adequately treated volunteers between the ages of 18 and 32, approximately 15 per cent failed to recover completely within 4 months, although all achieved complete clinical recovery within one year (2, 3). On the basis of these data and the high incidence of acute virus hepatitis during recent years, the occurrence of a relatively large number of such chronic cases (as defined above) might be expected.

Chronic hepatitis presents a number of important medical problems. Recognition frequently may be difficult, particularly in the non-icteric cases, and a lack of knowledge of the factors leading to chronicity has prevented a fundamental approach to its prevention and treatment. Furthermore, the chronic form of the disease may be of importance in the epidemiology of virus hepatitis, particularly when unrecognized.

The relationship of the hepatitis virus to the chronic stage of the disease unfortunately is obscure. A knowledge of this relationship obviously is essential for the clarification of some of the problems mentioned. For this reason, it was considered desirable to attempt to isolate the hepatitis virus from patients with a chronic form of the disease. Suitable materials for study were available from three volunteers with chronic active non-icteric hepatitis observed among a group of volunteers in whom acute infectious hepatitis had been induced under experimental conditions by the oral administration of feces known to contain the virus of infectious hepatitis (virus IH) described in previous reports (4, 5). It is the purpose of this preliminary report to describe the results of attempts to demonstrate the hepatitis virus in serum, feces, and liver tissue obtained from these three chronic cases.

MATERIALS AND METHODS

Source and materials. The histories of the three volunteers with chronic active non-icteric hepatitis have been described elsewhere (3) and do not warrant repetition here. The diagnosis appeared to be unequivocal and was based on the history, symptoms, laboratory evidence of hepatic disturbance, and, in two of the cases, on the histologic findings in liver biopsy specimens obtained 6 and 9 months respectively after the onset of acute hepatitis. The same strain of virus had been used for the induction of acute infectious hepatitis in all three cases. Specimens of feces and serum were collected at intervals through-

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[2] National Research Council Senior Fellow in the Medical Sciences.

[3] The term "virus hepatitis" is used to include both infectious hepatitis and homologous serum hepatitis.

out the course of the disease. The times of collection of the individual serum and feces specimens included in the pools used in the present study are shown in Figures 1, 2, and 3 in which the results of serial hepatic tests roughly reflect the course of the disease in the three cases. The liver tissue tested for the presence of virus was part of a biopsy specimen obtained surgically from Case 2 (HPZ) at the time indicated in Figure 2 (approximately 6 months after the onset of hepatitis). Serum and feces specimens were frozen within 1 hour after collection. They were stored at $-10°$ to $-20°$ C. until the time of administration to volunteers, with the exception of a few hours

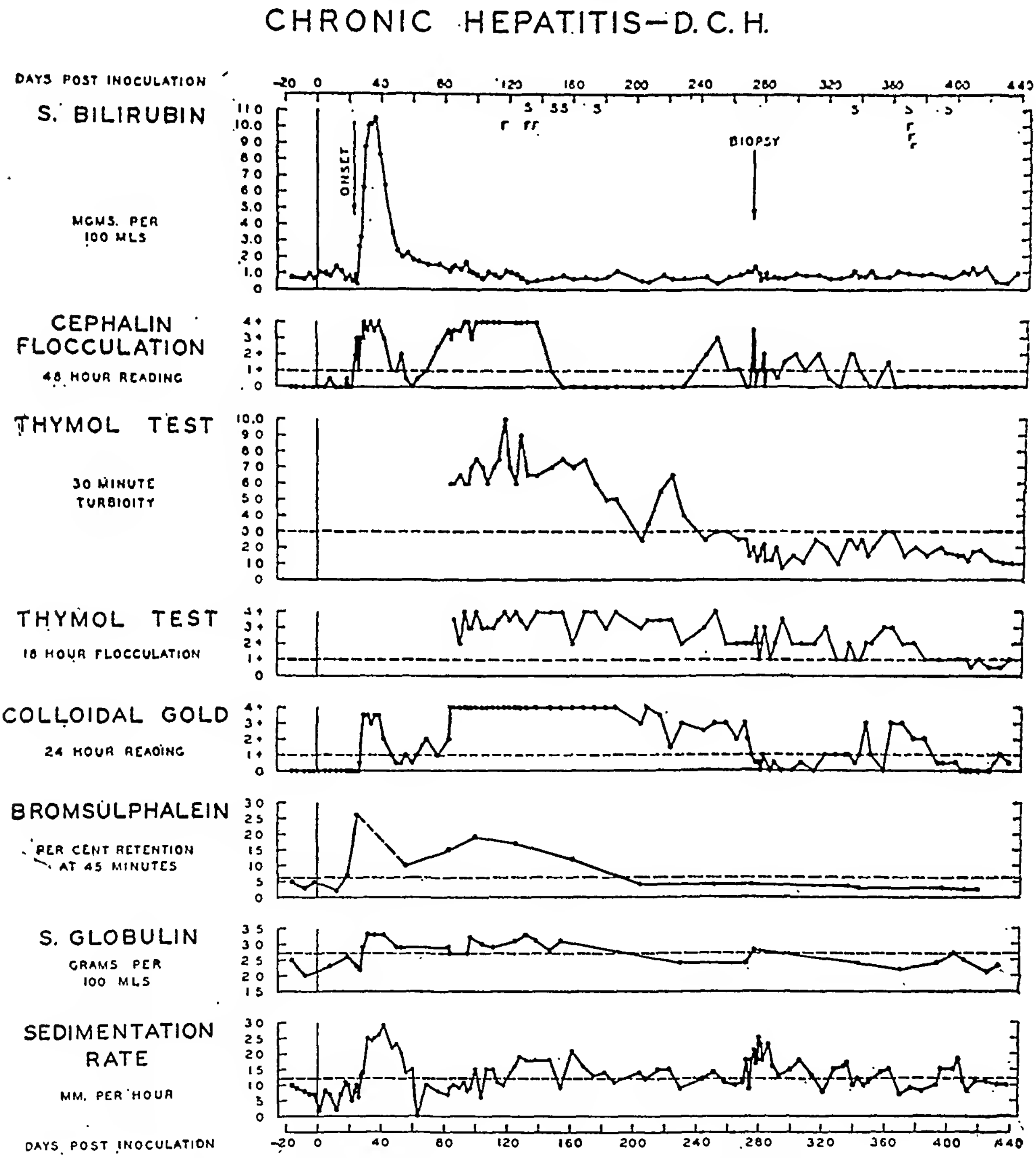

Fig. 1. Results of Serial Hepatic Tests in Volunteer D. C. H.

The "S" and "F" located beneath the time scale at the top of the figure indicate the times when serum (S) and feces (F) specimens were collected. Acute hepatitis had been induced in this subject by the oral administration of a feces preparation known to contain a representative strain (virus IH, Pa.) of infectious hepatitis virus. For approximately one year after the disappearance of jaundice associated with the acute hepatitis, he had persistent symptoms resulting in partial incapacitation. A microscopic examination of the liver biopsy, obtained at the indicated time, revealed the stroma of many of the portal triads to be infiltrated with excessive lymphocytes, plasma cells, and histiocytes.

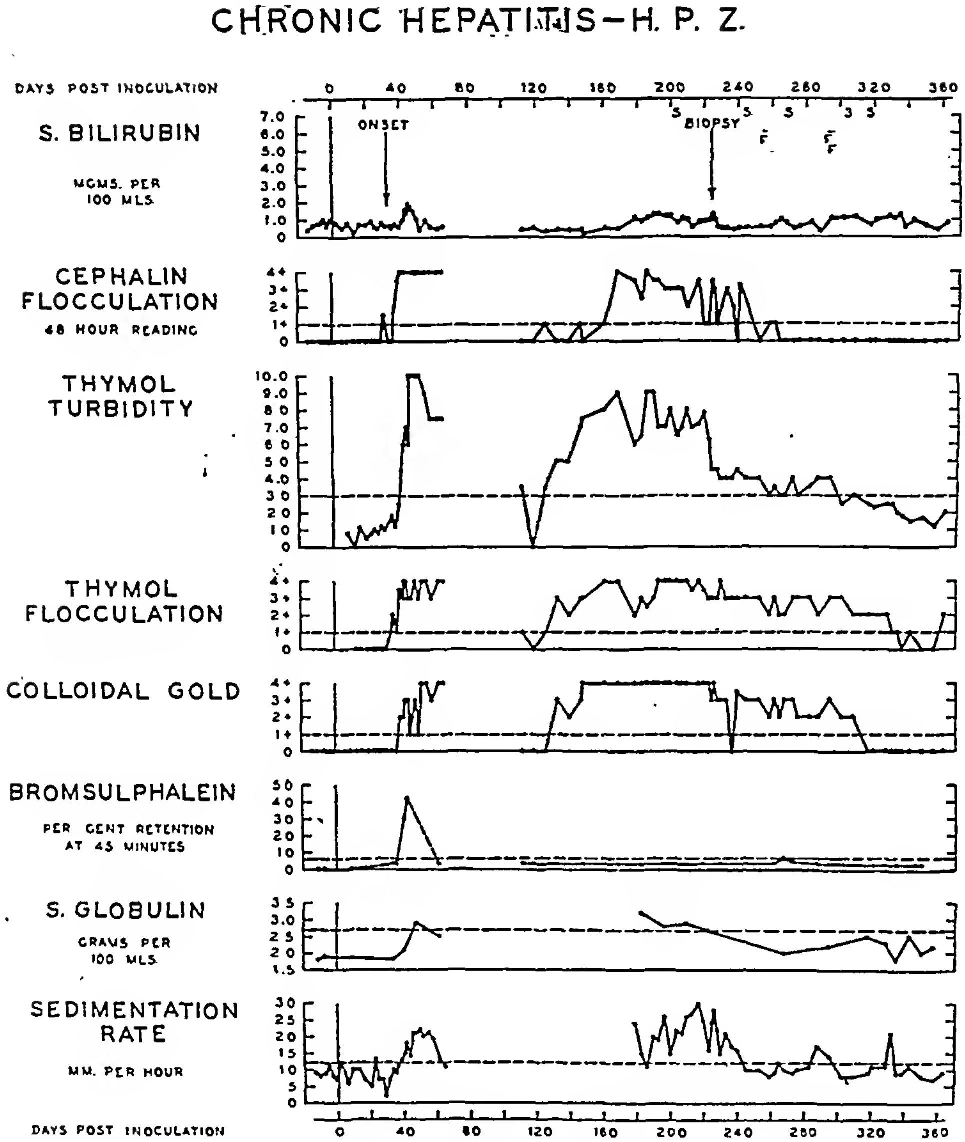

FIG. 2. RESULTS OF SERIAL HEPATIC TESTS IN VOLUNTEER H. P. Z.

Acute hepatitis was induced as described in the legend of Figure 1. For other data referable to the figure, see legend of Figure 1. A part of the liver biopsy obtained at the time indicated was used in the present studies (see text). This volunteer continued to have symptoms and partial disability for approximately 10 months after the onset of the acute attack.

on one day when they were thawed simultaneously, the pools prepared, and then refrozen. The liver tissue was placed in a dry bottle and was frozen within 15 minutes after its surgical removal. It also was stored at $-10°$ to $-20°$ C. until used.

Preparation of materials: Feces pool 13-14-15 FIH. The feces specimens from the individual cases were thawed and a portion of each was pooled with the others from the same case, yielding pools 13, 14, and 15 respectively. Sufficient distilled water was added to each of these pools to produce, after mixing, the consistency of a thick paste. Additional distilled water then was added and a heavy homogeneous suspension was obtained by thorough mixing in a Waring Blendor. This then was strained through gauze to remove any remaining large particles. Each of the 3 pools (13, 14, 15) then was cultured (no commonly pathogenic intestinal bacteria were isolated), and, after the culture reports were available, equal portions from each were pooled and mixed to yield *feces pool 13-14-15 FIH.* The final pool thus included a total of 23 feces specimens obtained 92 to 342 days after the initial onset of hepatitis (Figures 1, 2, and 3.).

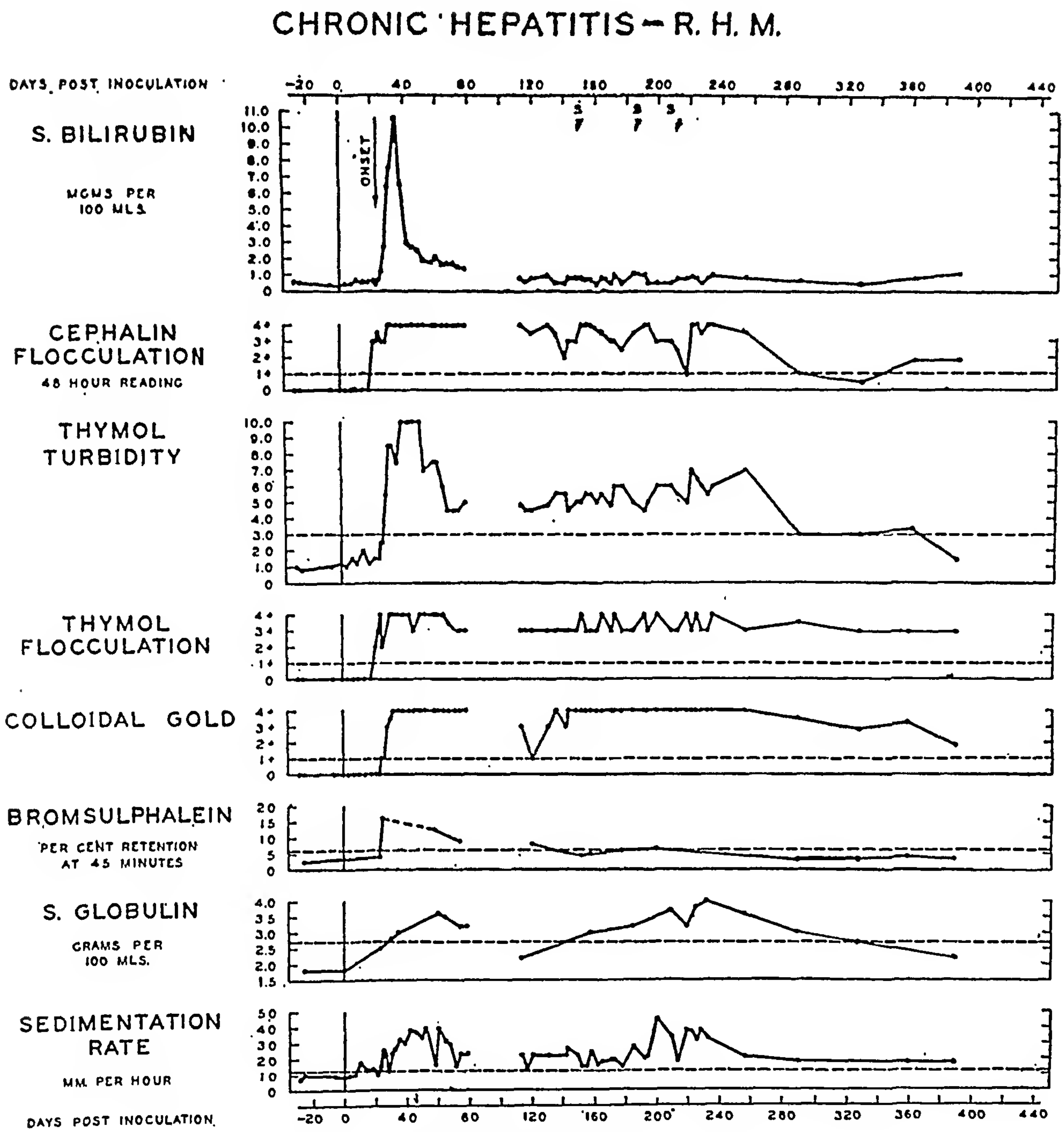

FIG. 3. RESULTS OF SERIAL HEPATIC TESTS IN VOLUNTEER R. H. M.

See legend Figure 1 for the other data referable to this figure. This subject continued to have persistent symptoms and partial disability for approximately 10 months after the onset of acute hepatitis.

Serum pool 1-CSIH. Approximately 2 ml. of each serum specimen obtained from 106 to 367 days after the onset of hepatitis were mixed together to form this pool which included a total of 15 serum specimens (Figures 1, 2, and 3).

Liver suspension 1-CLIH. The biopsy specimen of the liver tissue, estimated to weight between 100 and 200 mgm., was thawed and promptly was ground with sterile alundum. The ground liver tissue then was suspended in approximately 12 ml. of sterile beef heart infusion broth. The suspension was centrifuged for 10 minutes at 2,000 r.p.m. The supernatant then was removed and stored at − 10° C.

Just prior to its oral administration to the volunteers, the supernatant was thawed and diluted to approximately 100 ml. with sterile physiological saline solution and this (liver suspension 1-CLIH) was divided into equal portions (20 ml.) which were ingested by each of the 5 subjects.

The report of Colonel Balduin Lucké, of the Army Institute of Pathology, Washington, D. C., on the microscopic appearance of this liver tissue was as follows:

"The hepatic structure is preserved but allowance must be made for variations in lobular configuration that are normal at the surface of the liver. The stroma of most portal triads contains a moderate excess of cells, mostly lymphocytes and histiocytes; some triads, however, are entirely normal. No connective tissue proliferation is evident. Occasionally minute foci of cell reaction similar to those of the triads are met within the interior of some lobules. Many liver cells are swollen and appear to be laden with glycogen; these cells are often binucleated. It is probable that they represent regenerated cells. About half of the liver cells seem to have a low glycogen content, suggesting that storage of this material has varied in different parts of the tissue."

Volunteers. All of the men who volunteered as subjects for the study were male inmates of the New Jersey State Prison, Trenton, N. J. Because of statistically significant evidence (2) indicating that susceptibility to the virus of infectious hepatitis used in this study decreased after the age of 26, and decreased rapidly after the age of 30, an effort was made to obtain volunteers under 30 years of age. Unfortunately, only a few of the volunteers within this age range could be made available at the time. It was necessary, therefore, to use a number of men who were not within the age range of greatest susceptibility (up to 26 years of age) to this strain of virus. The ages of the 5 men who were inoculated with the feces pool were 23, 27, 31, 32, and 33 respectively. Those inoculated with the serum pool were 20, 29, 31, 32, and 39 respectively. Those who received the liver suspension were 21, 28, 29, 34, and 34 respectively. All were considered to be in good health. None had a history of previous jaundice or presented clinical evidence of hepatic disease prior to inoculation. All of the men were isolated as a group in a special ward provided by the New Jersey State Hospital, Trenton, N. J. The period of isolation began 1 month prior to inoculation and continued for 2 months after inoculation. During this period, the only contacts were with the nurses, the special guards assigned by the prison, the technicians, and the supervising physicians. None of these contacts reported any illness during the period of experiment. Although personal contact between the volunteers was not preventable at meal and recreation times, they were quartered in individual rooms with individual toilet facilities and individual eating utensils were used. Registered nurses were in constant attendance during the isolation period. Following their release from the isolation ward at the New Jersey State Hospital, the men were returned to the New Jersey State Prison where they were followed for an additional 3-month period.

Studies on volunteers. The hepatic studies described in previous reports (3, 6, 7) were conducted on each subject at frequent intervals both before and after inoculation. Total and prompt direct-reacting (1') serum bilirubin determinations, urine bilirubin and urobilinogen tests, and the cephalin-cholesterol flocculation, colloidal gold, and thymol tests were conducted at least twice weekly during the period of observation. Bromsulphalein tests were done once weekly. Serum total protein, albumin, and globulin were determined at less frequent intervals. Oral temperatures were recorded at least twice daily. With the appearance of any symptoms or laboratory abnormalities, the studies were repeated with greater frequency. After release from the isolation ward, the hepatic tests were continued at 1- to 3-week intervals.

Inoculation of volunteers. On each of 4 successive days, 5 ml. of feces pool 13-14-15 FIH was administered orally in chocolate milk to each of 5 volunteers. Each of 5 other volunteers ingested 3 ml. (1 man) to 5 ml. (4 men)

TABLE I

Results in volunteers after ingestion of feces pool 13–14–15 FIH

Subjects	Age	Illness (– to ++++)	D. A. I.	Remarks
J. D.	31	+	20 to 28	Symptoms only
		+++	127 to 137	Symptoms, liver tenderness, mild hepatic dysfunction
R. A.	32	+	18 to 41	Intermittent symptoms
G. S.	23	+	20 to 35	Symptoms only
E. S.	27	–	–	–
A. M.	33	–	–	–

Illnesses graded from – (no illness) to ++++ on the basis of the relative severity of symptoms and signs as observed in these subjects. D.A.I. indicates the number of days after inoculation when symptoms were present.

TABLE II

Results in volunteers after ingestion of liver suspension 1–CLIH

Subjects	Age	Illness (– to ++++)	D. A. I.	Remarks
E. Mc.	29	+	22 to 29	Symptoms
		++++	36 to 50	Symptoms, plus hepatic enlargement and tenderness; no significant hepatic dysfunction detected
T. K.	21	+	26 to 30	Symptoms and laboratory evidence suggestive of mild hepatic dysfunction
M. P.	28	+	28 to 51	Intermittent symptoms only
P. S.	34	+	15 to 60	Intermittent symptoms only
J. M.	34	–	–	–

See legend of Table I for other data referable to interpretation of table.

TABLE III

Results in volunteers after ingestion of serum pool 1-CSIH

Subjects	Age	Illness (− to ++++)	D. A. I.	Remarks
V. P.	39	±	10 to 15	Very mild symptoms only
E. S.	31	±	14 to 26	Very mild symptoms only
W. H.	32	−	−	−
A. W.	20	−	−	−
L. S.	29	−	−	−

See legend of Table I for other data referable to interpretation of table.

of the undiluted serum pool 1-CSIH, the material being administered in milk. The 5 men of the third group each ingested (in milk) 20 ml. of liver suspension 1-CLIH.

Previous experience with the strain of infectious hepatitis virus (virus IH) that was used for induction of hepatitis in the three volunteers from which the present materials were obtained had shown it to be highly effective in inducing the apparent disease when administered orally but much less effective when administred parenterally. For this reason, and because of the limited number of volunteers available for these preliminary studies, only the oral route of inoculation was used.

RESULTS

The results obtained from the oral administration of the feces, liver, and serum preparations are summarized in Tables I, II, and III and are described in detail below.

Feces pool 13–14–15 FIH

J. D. (AGE 32). Eleven days after inoculation he complained of cramps in the abdomen. On the 16th day, he noted a "slight rash" associated with pruritis. As this was transient, it was not observed by a physician and a reliable description is not available. From the 20th to 28th days inclusive, he complained of nausea, headache, and general malaise. No significant physical findings were recorded during this period, and no definite evidence of hepatic disturbance was revealed by the various hepatic tests. After the 28th day, he apparently was well until the 127th day. He had been released from the isolation ward and returned to the New Jersey State Prison on the 70th day. On approximately the 122nd day, he was released from the prison and went to his home. From the 122nd to 127th days, he had little sleep and ingested moderate quantities of alcoholic beverages. On the 127th day, he noted extreme malaise. This was associated with chilly sensations and his temperature was found to be 102.8° F. (oral). He was admitted the next day (128th) to the Hospital of the University of Pennsylvania. At that time, he complained chiefly of weakness, headache, aching of the eyes on movement, anorexia, and pain in the right costovertebral angle. Examination revealed an oral temperature of 102° F., slight enlargement of the inguinal, axillary, and cervical lymph nodes, and pronounced tenderness in the right upper quadrant and right costovertebral angle. The liver and spleen were not palpable. The symptoms and findings persisted until the 132nd day after which they gradually subsided. By the 137th day, he was asymptomatic and physical examination was negative. The oral temperature varied between 99° and 101° F. on the 129th and 130th days, from normal to 100.4° F. on the 131st and 132nd days, and thereafter was normal. He was discharged from the hospital on the 137th day.

The results of the laboratory studies made during this period were as follows: The blood hemoglobin varied between 90 and 95 per cent; the total leukocyte count was 5,000 on the 128th day and 5,000 on the 131st day. The differential leukocyte count on the 131st day revealed: neutrophiles—59 per cent, lymphocytes—40 per cent, monocytes—1 per cent. Routine urinalysis consistently revealed no abnormal findings. The *hepatic* tests gave the following results suggesting the existence of mild hepatic disturbance: The Harrison spot test for urine bilirubin was constantly positive between the 128th and 139th days, the response varying from 1 to 3 +. Urine urobilinogen was somewhat increased, the maximum recorded value being 2.0 Ehrlich units in a morning 2–hour specimen. Bromsulphalein tests on the 128th, 129th, 130th and 137th days gave 30- and 45-minute values (30 minute per cent retention —45 minute per cent retention) of 7/4, 12/11, 7/4, and 4/3 respectively. The results of the total and prompt direct reacting serum bilirubin determinations, the cephalin-cholesterol flocculation, collodial gold and thymol, and the serum pro-

tein and cholesterol studies showed no significant variations from the normal.

Typhoid H and O, paratyphoid A and B, and brucella abortus agglutinations on the 126th day were negative. A heterophile antibody test (Paul-Bunnell) on the 137th day (maximal response graded 4 +) gave 2 +, 1 +, and negative reactions in the 1:32, 1:64, and higher dilutions respectively.

Although very cooperative, this subject was anxious to leave the hospital as soon as possible due to the fact that he had been released from prison just prior to onset of this illness. As he felt perfectly well by the 137th day, it was not possible to detain him for further studies after that time. Two months later, he returned for follow-up studies, having been entirely well during the interval. All hepatic tests were normal and the heterophile antibody test gave a 1 + reaction in the 1: 64 dilution, the reaction being negative in the higher dilutions.

R. A. (AGE 32). Eighteen days after inoculation, this subject complained of general malaise and moderate pain in the lumbar area of the back. Between the 19th and 22nd days, he voluntarily remained in bed and complained of malaise, backache, and anorexia. On the 23rd day, he had intermittent chilly sensations during the evening. These continued during the 24th and 25th days and were associated with nausea and one episode of vomiting. Although he then felt relatively normal until the 39th day, his oral temperature varied from 99.8° to 100.8° F. between the 34th and 37th days. From the 39th to the 41st day, he complained of persistent headache and intermittent nausea. After the 41st day, he remained asymptomatic. No significant physical findings, other than the slight elevation of temperature, were recorded. The hepatic studies revealed no definite evidence of hepatic disturbance. However, the thymol test, which had not given 30-minute turbidity readings greater than 1.5 units or 18-hour flocculation reactions greater than 1 + with numerous serum specimens obtained during the month prior to inoculation, gave turbidity readings up to 3.0 units and 2 + flocculation reactions during the period of symptoms.

G. S. (AGE 23). On the 11th day, the subject complained of slight nausea, headache, and abdominal cramps that persisted for only 24 hours. From the 20th to the 35th days, he had frequent headaches, intermittent lumbar backache, and mild to moderate anorexia and nausea. On the 25th and 30th days, he vomited several times. The only laboratory abnormality was the finding of an increased excretion of urine urobilinogen (2.0 E. U.) on the 34th day. He remained well after the 35th day.

SUBJECTS E. S. (AGE 27) AND A. M. (AGE 33). The remaining two volunteers who ingested feces pool 13-14-15 FIH experienced no significant symptoms, and no evidence of hepatic disturbance was revealed by hepatic tests during the period of observation.

Liver suspension (1–CLIH)

E. MC. (AGE 29). This subject was well until the 22nd day when, for a period of 24 hours, he reported general malaise, anorexia, nausea, and one episode of vomiting. Although he was relatively asymptomatic between the 23rd and 26th days, symptoms recurred on the 26th day, and persisted till the 29th day. During this period he complained of weakness, general malaise, anorexia, nausea, and pain in the region of the right costovertebral angle. He was relatively free from symptoms between the 29th and 36th days. However, from the 36th to the 50th day, he experienced rather severe symptoms. They included: (1) marked malaise of sufficient intensity to keep him in bed; (2) complete anorexia, severe nausea, and vomiting one or more times daily for a period of approximately 10 days; any attempt to eat solid food was followed by vomiting; (3) headache with considerable orbital pain on movement of the eyes; (4) pain in the right upper quadrant that at times was of sufficient severity to require morphine. He was given daily intravenous infusions of glucose and saline during this period. No significant temperature elevations were recorded until the 48th day when the oral temperature was 100° F. It fluctuated between 98° and 100° F. on the 48th and 49th days. About 2 hours after a plasma transfusion on the 50th day, he had a chill and, during the next few hours, his temperature gradually rose to a maximum of 107° F. (rectal). The significance of the fever noted on the 48th and 49th days is uncertain as he had received glucose and saline infusions on these days. That of the 50th day appeared to be the result of

a plasma reaction. With the subsidence of this reaction, all of his symptoms diminished rapidly and he felt relatively normal by the 65th day.

Physical examination throughout the period of severe symptoms (36th to 50th days) revealed marked tenderness in the right upper quadrant. The liver was enlarged and quite tender. With inspiration, the edge of the liver was easily palpable 3 to 4 cm. below the costal margin. After subsidence of the symptoms, the size of the liver decreased. The spleen was not palpable. No other significant physical abnormalities were detected.

The several blood counts (hemoglobin, total and differential leukocyte counts) made during the period of acute symptoms revealed no significant abnormalities. Hepatic tests revealed no significant abnormalities until the 49th day when the urine gave a positive reaction for urobilinogen in a dilution of 1 to 30 and bromsulphalein retention was 10 per cent at 30 minutes and 3 per cent at 45 minutes.[4] The following day the 30- and 45-minute retentions were 40 and 30 per cent respectively. The urine was positive for urobilinogen in dilutions of 1 to 100 and 1 to 150 on the 50th and 51st days. On the 55th day, difficulty was encountered in obtaining a 30-minute blood specimen for the bromsulphalein test. A specimen obtained at 41 minutes revealed 8 per cent retention. The other hepatic tests revealed no significant abnormalities and thereafter all results were within normal limits. The abnormalities mentioned are of doubtful significance in respect to his illness because of their relationship to the reactions from the intravenous infusions. Roentgenological examination of the gastrointestinal tract on the 57th day revealed no abnormal findings.

T. K. (AGE 21). Between the 26th and 30th days after inoculation, he complained of malaise, mild anorexia, nausea, one episode of vomiting, and headache. These subsided by the 30th day and he remained well thereafter. There were no abnormal temperature elevations.

The hepatic tests during this period revealed a maximum total serum bilirubin of 1.7 mgm. per 100 ml. (pre-inoculation maximum 1.2 mgm.), a delayed direct qualitative van den Bergh reaction, a maximum thymol turbidity of 4.0 units (maximum pre-inoculation reading—2.5 units), and a maximum 2-hour urine urobilinogen concentration of 6.0 Ehrlich units (maximum pre-inoculation 1.6) with a positive reaction also being obtained in a urine dilution of 1 to 200.

J. M. (AGE 34). This subject had no significant symptoms or laboratory manifestations of hepatic disturbance following inoculation.

M. P. (AGE 28). Between the 28th and 51st days following inoculation, he complained of malaise and intermittent rather severe headaches. No other significant symptoms or laboratory manifestations suggestive of hepatic disturbance were present.

P. S. (AGE 34). Fifteen days after inoculation a pruritic rash, slight headache, and an elevation of oral temperature to 100° F. were experienced. No adequate description of the rash is available as it was observed only by the attending nurse and disappeared in 24 hours before he was seen by a physician. From the 21st to 26th days he was nauseated intermittently, vomited on two occasions, and his appetite was moderately diminished. He then was relatively asymptomatic until the 39th day when anorexia, abdominal cramps, and headache were present and persisted for approximately 2 days. He again was free from symptoms until the 59th day when, for a 24-hour period, he had anorexia, nausea, and one episode of vomiting. Thereafter he remained well. No significant laboratory evidences suggestive of hepatic disturbance were detected at any time.

Serum pool (1–CSIH)

None of the 5 men who ingested this serum (V. R., age 39; E. S., 31; W. H., 32; L. S., 29; A. W., 20) developed significant laboratory evidence suggestive of hepatic disturbance following their inoculation. One (V. P.) complained of slight nausea and mild headache between the 10th and 15th days. Another (E. S.) between the 14th and 26th days, had malaise, and intermittent mild nausea and headache. The symptoms in both were not sufficient to interfere with their voluntary continuance of their usual activities.

[4] With 7 previous bromsulphalein tests performed during the preceding 2 months, retention at 30 and 45 minutes was never greater than trace and O respectively.

DISCUSSION

The results of the present studies permit no definite conclusions concerning the presence of a hepatitis virus in the serum, feces, and liver preparations. Nevertheless, four of the five men who ingested the liver suspension and three of the five who ingested the feces preparation developed illnesses, of mild to moderate severity, after intervals corresponding to the usual incubation period of infectious hepatitis. The symptoms of subject E. McE. (liver suspension) were typical of infectious hepatitis of moderate severity and were associated with enlargement and tenderness of the liver. The only definite laboratory evidence of hepatic disturbance in this subject was observed late in the course of the illness. This was quite transient and could be explained entirely on the basis of febrile reactions that followed intravenous glucose, saline, and plasma. Except for the lack of laboratory evidence of hepatic disturbance, his illness clinically was typical of infectious hepatitis without jaundice.

The symptoms and physical findings in volunteer J. D. (feces preparation) beginning 127 days after inoculation also were typical of mild infectious hepatitis without jaundice. Liver tenderness and laboratory evidence of mild hepatic disturbance were present. This illness began after a period of unaccustomed physical activity, inadequate rest, and ingestion of considerable amounts of alcohol, all following his release after a number of years in prison. Although the possibility that this illness was coincidental and unrelated to the experiment obviously cannot be excluded, it also is quite possible that his symptoms beginning on the 20th day were the result of the hepatitis virus and that his resistance was sufficient to protect him until it was lowered by the activities following his release from the prison. Such activities have been related to an increased incidence of relapse in infectious hepatitis.

The illnesses of the other men were mild. The symptoms and the interval between inoculation and their onset were similar to the symptoms and incubation period of infectious hepatitis. However, evidences of hepatic disturbance either were absent or minimal. In other volunteers with symptoms of comparable and even less severity due to hepatitis resulting from inoculation with this strain of virus, as obtained from acute cases, the illness usually has been associated with fever, and hepatic tests consistently have revealed evidence of marked hepatic disturbance. If these illnesses are considered to be due to a hepatitis virus in the materials obtained from the volunteers with chronic hepatitis, the difference in the manifestations observed in these volunteers from those observed in others inoculated with materials from acute cases must be explained. Some change in the virus itself, a difference in the resistance of the volunteers used, or both possibly could account for the difference. Unfortunately, the volunteers available at the time of the present experiment did not include persons in the age groups most susceptible to the strain of virus concerned. Previous studies (2) have shown that the age range of greatest susceptibility to this strain of virus was from 6 to 25, the incidence in exposed persons over this age being much lower. Furthermore, with the more marked decrease in incidence observed after the age of 30, the incidence of hepatitis without jaundice in those over this age who acquired the disease increased from 26 per cent in the 10 to 30 age group to 66 per cent of those over 30. Of the 15 volunteers available for the present study, only 3 (1 in each group of five) were under 25, 4 were between 25 and 30, and 8 were over 30 years of age. It is possible, therefore that the illness observed in these volunteers represented the response of relatively resistant hosts to the hepatitis virus. On the other hand, it also is possible that, in the volunteers with chronic non-icteric hepatitis, some modification of the virus had occurred. However, lacking any specific serological or other test for the hepatitis virus, conclusions regarding the significance of the present results must be deferred. It is hoped that additional volunteers will become available for further tests of the materials used in the present study and of materials obtained from the present subjects during their etiologically obscure illnesses. In addition to the possible influence of factors mentioned above, the failure of serum pool 1-CSIH to produce any significant manifestations suggesting the presence of the hepatitis virus also could have been due either to the absence of the virus in the pool or to the presence, in some of the specimens, of neutralizing substances which inactivated the virus. Thus, the

negative results with this serum pool do not necessarily indicate that the virus was absent from all of the individual specimens.

SUMMARY AND CONCLUSIONS

Serum and feces specimens and liver tissue (biopsy) were obtained from 3 subjects who had persistent symptoms and other evidence of continued hepatic disturbance (chronic nonicteric hepatitis) following acute hepatitis that had been induced by oral inoculation with a known strain of "infectious hepatitis" virus. In an attempt to clarify the relationship of the hepatitis virus to the persistent symptoms and continued hepatic disturbance, volunteers were inoculated orally with materials collected from 3 to 12 months after the onset of the disease. Following ingestion of the feces preparation, 3 of the 5 volunteers developed mild illnesses after 18 to 20 days. Likewise, 4 of the 5 volunteers developed illnesses of mild to moderate severity 15 to 36 days after ingestion of a suspension of the liver tissue. None of the 5 volunteers who ingested the serum pool developed significant illness during a 5-month period of observation. The symptoms and manifestations of those who became ill included two or more of the following: malaise, headache, anorexia, nausea, vomiting, abdominal cramps, and pain in the right upper quadrant. Two presented tenderness in the liver area and, in one of these, the liver became moderately enlarged.

In spite of moderately severe symptoms in two of the cases, laboratory evidences of hepatic disturbance in all cases either were absent or minimal. None developed overt jaundice. The intervals between inoculation and onset and the clinical manifestations were compatible with mild infectious hepatitis without jaundice, particularly in two of the cases. The laboratory evidences of hepatic disturbance usually associated with mild hepatitis, however, were not present. Although no other explanation for the illnesses is apparent, their exact nature and their relationship to the materials used for inoculation are uncertain. Factors of possible importance in interpretation of the results are discussed.

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BIBLIOGRAPHY

1. Barker, M. H., Capps, R. B., and Allen, F. W., Chronic hepatitis in the Mediterranean theater. J. A. M. A., 1945, 129, 653.
2. Neefe, J. R., Recent advances in the knowledge of virus hepatitis. Med. Clin. N. America, 1946 (November—Philadelphia No.), 1407.
3. Neefe, J. R., Results of hepatic tests in chronic hepatitis without jaundice. Gastroenterology, 1946, 7, 1.
4. Neefe, J. R., and Stokes, J., Jr., An epidemic of infectious hepatitis apparently due to a water borne agent. J. A. M. A., 1945, 128, 1063.
5. Neefe, J. R., Gellis, S. S., and Stokes, J., Jr., Homologous serum hepatitis and infectious (epidemic) hepatitis; studies in volunteers bearing on immunological and other characteristics of the etiological agents. Am. J. Med., 1946, 1, 3.
6. Neefe, J. R., Stokes, J., Jr., and Gellis, S. S., Homologous serum hepatitis and infectious (epidemic) hepatitis; experimental study of immunity and cross immunity in volunteers, a preliminary report. Am. J. Med. Sci., 1945, 210, 561.
7. Neefe, J. R., and Reinhold, J. G., Laboratory aids in the diagnosis and management of infectious (epidemic) hepatitis; analysis of results obtained in 34 volunteers during the early and convalescent stages of induced hepatitis. Gastroenterology, 1946, 7, 393.

BLOOD LACTATE RESPONSE DURING MODERATE EXERCISE IN NEUROCIRCULATORY ASTHENIA, ANXIETY NEUROSIS, OR EFFORT SYNDROME [1]

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INTRODUCTION

It has been amply demonstrated that the concentration of blood lactate rises during moderate exercises (1). It was therefore of interest to study the response of blood lactate in men with neurocirculatory asthenia (N.C.A.), anxiety neurosis, or effort syndrome to a standard amount of moderate exercise. Studies have been made on blood lactate response in patients with effort syndrome (2). In those studies, however, the patients exercised a lesser amount than did the control subjects, thereby rendering the blood lactate results difficult of interpretation. Two types of work experiments must be differentiated. The first is that in which the patient is asked to perform until exhaustion. In this type of experiment, it is of interest that one subject may perform longer than another. Physiologic data from this type of study are difficult to compare with those of control subjects since time of exercise is not comparable (3, 4). This type of experiment is subject to considerations of the subjects' willingness to perform, "true ability" to perform, persistence, and similar factors which are as yet difficult to quantitate.

Another type of work experiment is the moderate exercise test. The task is set at a level where the work can be finished both by the patient and the control subject. This study reports tests of this second kind. By using a standard amount of work which both the patient and control subject finish it is possible to compare response of one group of patients with a control group.

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital. Responsible Investigators: Paul D. White and Stanley Cobb.

METHOD

Patients selected were men in the armed forces in whom a diagnosis of neurocirculatory asthenia had been made (5). Forty-two patients with chronic neurocirculatory asthenia and 19 patients with acute neurocirculatory asthenia were studied, along with 41 healthy control subjects. On grounds of history alone, two groups of patients were differentiated. Those who had a lifelong course of the condition or who could never do hard work or athletics were designated "chronic neurocirculatory asthenia," while those who gave convincing evidence of good health, ability to do muscular work or athletics, and nervous stability previous to the onset of the illness were designated "acute neurocirculatory asthenia." The control subjects were soldiers who had been through basic training and were in a good state of health. These studies were done as part of a general study of work in neurocirculatory asthenia. A half-hour rest period is followed by a 15-minute walk on a treadmill, which is set at 3.5 miles per hour, and 8.9 per cent grade. The work output in this test is 1.17 large calories per kilo and hour (81.90 large calories per hour for a 70-kilo man). Specimens of finger blood are collected during the ninth minute of the walk, blood being taken from the finger of the dependent hand into a heparinized watch glass. Care is taken to avoid contaminating the blood with alcohol or any other substance which might interfere with the accurate estimation of blood lactate. The determinations were done in duplicate for lactate concentration by the method of Edwards (6). The values recorded here are the mean for duplicate determinations which checked within 3 milligrams. In some cases respiratory studies (ventilation and O_2 consumption) were done from the ninth to the fourteenth minute of the walk. The walk is concluded at the end of 15 minutes. Patient is given a short rest and then allowed to run on the treadmill to exhaustion. The results of the run will not be reported on or discussed in detail in this paper (7).

RESULTS

All patients reported here finished the full walk. Results of blood lactate concentration determinations in the patients are presented in Figure 1 which shows that patients' average is higher than

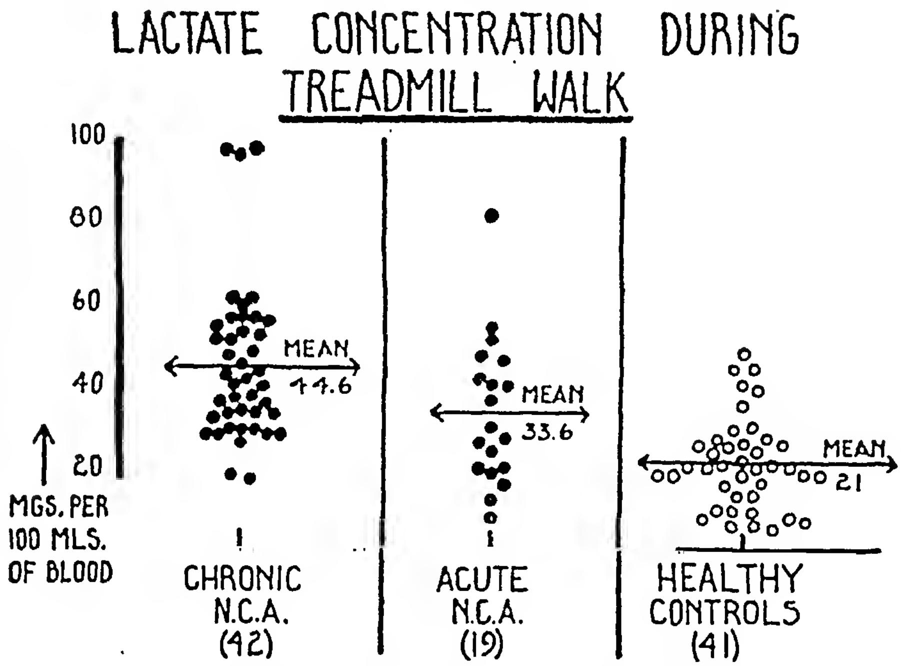

FIG. 1. DATA FOR BLOOD LACTATE CONCENTRATION DURING TREADMILL WALK

Each dot or circle represents average of duplicate determinations from first test in each subject. The patients show significantly higher blood lactate concentration as compared with controls. The chronic N.C.A. patients show significantly higher levels of blood lactate concentration as compared with acute N.C.A.

that of healthy control subjects. The men with acute neurocirculatory asthenia do not average so high as do the men with chronic neurocirculatory asthenia. The differences between these groups are statistically significant as is shown in Table I. The mean blood lactate concentration for all 61 N.C.A. patients is 41.2 mgm. per 100 ml. of blood. This differs markedly from the controls with a significance ratio of the difference being 7.02.

TABLE I

Summary of averages, statistical calculations, and significances of differences in lactate results in walking

	Healthy control subjects	Acute N.C.A.	Chronic N.C.A.
Number of cases	41	19	42
Mean	21.3	33.6	44.6
Coefficient of variation	.51	.53	.42
Standard deviation	10.86	17.86	18.52
Standard error	1.70	4.10	2.86
Standard error of the difference between means:			
Healthy		4.44	3.32
Acutes	4.44		5.00
Chronics	3.32	5.00	
Significance ratio: Difference of means/standard error of the difference between means:			
Healthy		2.79	7.02
Acutes	2.79		2.20
Chronics	7.02	2.20	

Odds against the occurrence of a deviation as great or greater than the designated number of Standard Errors:
Healthy to acutes—180 to 1. Acutes to chronics—35 to 1. Chronics to healthy—400,000,000,000 to 1.

Table II shows the resting blood lactate values along with the values obtained during walking for each subject. The resting values for the controls and the patients are the same despite the fact that the walking values are higher for the patients.

There is no correlation in individual cases between resting and walking values of blood lactate concentration in N.C.A. or controls (Correlation Coefficients, chronic N.C.A., .25; healthy, .18).

Table III shows that replicate determinations, from successive walk studies in patients with N.C.A., give consistent results, the difference in means of the two groups being less than one mgm. per 100 ml. of blood.

TABLE II

Lactate concentration—mgm. per 100 ml. of blood

Chronic N.C.A.			Acute N.C.A.			Healthy controls		
Case	Resting	Walking	Case	Resting	Walking	Case	Resting	Walking
3	14	57	201	20	40	401	13	27
5	18	33	204	19	41	406	10	20
7	16	28	206	10	47	407	15	16
11	—	47	207	11	16	410	19	44
13	18	33	213	12	12	411	20	30
14	8	97	215	18	27	416	14	20
18	18	36	216	18	37	417	15	44
21	26	33	217	22	24	418	18	26
22	16	61	221	11	30	422	19	20
23	12	29	223	12	8	424	14	18
24	24	52	224	12	51	430	10	39
25	—	54	225	16	26	435	26	25
28	10	32	230	10	39	436	20	40
30	—	48	231	9	19	438	16	24
31	—	28	238	13	81	439	13	19
32	18	53	239	11	40	440	9	24
33	—	29	240	—	54	442	19	26
34	12	37	241	12	20	445	21	48
38	14	56	248	9	20	447	21	26
39	19	61				448	13	20
40	14	39				450	14	29
41	15	40				451	11	18
44	9	27				452	—	18
45	13	44				455	22	18
46	—	34				490	—	6
49	30	97				491	—	8
52	—	18				492	—	7
55	—	42				493	—	9
58	11	17				494	—	5
61	16	51				495	—	9
63	9	59				496	—	21
65	23	56				497	—	21
67	13	26				498	—	35
68	14	41				499	—	7
70	12	37				500	—	6
71	45	45				501	—	9
73	13	51				502	—	22
74	24	36				503	—	15
80	—	28				504	—	13
86	11	41				505	—	13
87	—	29				506	—	27
89	13	55						
N	32	42		18	19		23	41
Mean	11.8	44.6		13.9	33.6		16.2	21.3

DISCUSSION AND SPECULATION

These data show that during standard moderate exercise blood lactate concentration in patients is greater than that of healthy control soldiers. Similarly, poorly trained subjects (8), normal women (9), and elderly men (1) show high blood lactate concentration during treadmill walking. These patients had spent some time in the hospital and were not in active training. Most of them had been through basic training in service. Whether these results merely reflect poor state of training cannot be ascertained from these data.

TABLE III

Blood lactate during successive walks on different days in patients with neurocirculatory asthenia

Case	First walk	Second walk	Difference
	mgm. per 100 ml. of blood		
21	33.0	41.0	8.0
28	32.2	31.0	− 5.5
34	37.0	29.0	− 8.0
40	39.0	36.0	− 3.0
44	27.0	30.0	3.0
48	17.0	11.0	− 6.0
63	59.0	87.0	28.0
73	51.0	50.0	− 1.0
74	36.0	30.0	− 6.0
206	47.0	37.0	−10.0
207	16.0	20.0	4.0
223	8.0	24.2	16.2
238	107.0	76.0	−31.0
No.	13	13	13
Mean	39.2	38.6	− 0.9

An attempt to train some of these men was unsuccessful as the men did not train properly (4). This left open the question as to whether training would bring this abnormality in blood lactate concentration to normal levels. Methyl testosterone, administered because of similarities in some aspects of the physiology of N.C.A. with that of women and older men, did not increase physical fitness test scores in N.C.A., nor did intravenous administration of cytochrome C change the blood lactate concentration during walk significantly (7). These data do not tell whether the high blood lactate in N.C.A. during walking is due to increased formation, increased blood concentration, abnormal rate of destruction, or removal from blood.

The abnormality in blood lactate concentration during moderate work shows a clear cut difference betwen N.C.A. and healthy control subjects. It suggests that N.C.A. patients have special difficulties in doing muscular work and fits well with patients' constantly reiterated statements that they are unable to keep up in muscular work with other men.

In discussing their lactic studies, Jones and Scarisbrick (10) concluded that the patients "show very poor response to exercise as judged by the amount of work done." At the same time, however, there is no major biochemical abnormality in their response. The pH and lactate were in keeping with the amount of work done, though we have ocasionally seen a case where the rise of

lactate and pH shift is excessive for a small output of work." This inspired a medical editorial which stated (11), "The patients' bodily response to exercise does not differ in any discoverable respect from that of other subjects of their own age. . . . The level of lactic acid reached in these experiments lent no support to the view that patients with effort syndrome behave otherwise in physiologic respects to other people."

Our data are in direct contradiction to these statements. It is true, to be sure, that if N.C.A. patients perform for a shorter time than do controls their blood lactate concentration may be lower. This was true in our studies of severe work where patient's ran for a shorter time (4). However, for the same duration and amount of muscular work, whether moderate or severe (4, 7, 12), blood lactate concentration is high in N.C.A. The lower level at "exhaustion" and the higher levels for the same amount and duration of muscular work confuse some readers and may seemingly offer a contradiction. The seeming contradiction is due to the fact that in severe muscular work N.C.A. patients reach the stopping point earlier than do controls; but, for the same amount and duration of effort, blood lactate concentration is higher in N.C.A. This demonstrates that, if the patients' difficulty is, as was stated (10), simply a matter of lack of "persistence," this lack of persistence is usually associated with a chemical abnormality—that is, increased blood lactate concentration.

The exact explanation for this is not at hand. Taken with our evidence of low oxygen consumption in N.C.A. during running (7, 12), it suggests deficient aerobic metabolism and excessive oxygen debt. It is noteworthy that cytochrome C did not correct the high blood lactate during walking in N.C.A. We have no data which explain completely the exact mechanism of this abnormality in neurocirculatory asthenia.

CONCLUSIONS

1. Blood lactate concentration is significantly higher in neurocirculatory asthenia during moderate exercise than in controls.

2. The abnormality is greater in chronic N.C.A. than in acute N.C.A.

3. It cannot be stated whether state of training or other factors account for the difference.

4. A chemical abnormality has been demonstrated in neurocirculatory asthenia which fits well with patients' statements that they cannot keep up with other men in muscular work.

BIBLIOGRAPHY

1. Robinson, S., Experimental studies of physical fitness in relation to age. Arbeitsphysiol., 1938, 10, 251.
2. Jones M., and Scarisbrick, R., Effort intolerance in soldiers; review of 500 cases. War Med., 1942, 2, 901.
3. Johnson, R. E., Brouha, L., and Darling, R. C., A test for physical fitness for strenuous exertion. Revue Canadienne de Biologie, 1944, 1, 491.
4. Cohen, M. E., Johnson, R. E., Cobb, S., Chapman, W. P., and White, P. D., Studies of work and discomfort in patients with N.C.A. J. Clin. Invest., 1944, 23, 934.
5. White, P. D., Cobb, S., Chapman, W. P., Cohen, M. E. and Badal, D. W., Observations on N.C.A. Tr. A. Am. Phys., 1944, 58, 129.
6. Edwards, H. T., A simplified estimation of lactate in normal human blood. J. Biol. Chem., 1938, 125, 571.
7. Cohen, M. E., Johnson, R. E., Chapman, W. P., Badal, D. W., Cobb, S., and White, P. D., A study of neurocirculatory asthenia, anxiety neurosis or effort syndrome. Final Report to the Committee on Medical Research of the Office of Scientific Research and Development under contract OEM-cmr-157, 1946.
8. Edwards, H. T., Brouha, L., and Johnson, R. E., Effets de l'entrainement sur le taux de l'acide lactique sanguin au cours du travail musculaire. Travail Humain, 1940, 8, 1.
9. Metheny, E., Brouha, L., Johnson, R. E., and Forbes, W. H., Some physiologic responses of women and men to moderate and strenuous exercise. A comparative study. Am. J. Physiol., 1942, 137, 318.
10. Jones, M. S., and Scarisbrick, R., Effect of exercise on soldiers with effort intolerance. Lancet, 1943, 245, 331.
11. Annotation on the biochemistry of effort syndrome. Lancet, 1943, 245, 329.
12. Cohen, M. E., Johnson, R. E., Consolazio, F., and White, P. D., Low oxygen consumption and low ventilatory efficiency during exhausting work in patients with neurocirculatory asthenia, effort syndrome, anxiety neurosis. J. Clin. Invest., 1947, 26, 920.

WATER STORAGE AND THE MOVEMENTS OF BODY FLUIDS AND CHLORIDES DURING ACUTE LIVER DISEASE

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The role of the liver in directing fluid shifts in the body has never been accurately defined. The effect of hepatic disease on the water balance of the body, however, has been appreciated for many years. In evidence of this is the fact that a patient with liver disease experiences greater delay in diuresis than does a normal person after drinking large amounts of water (1). The suppression of urine that may accompany acute liver disease as well as the oliguria of cirrhosis are old clinical observations (1, 2, 3, 4, 5). The phenomenon of water storage during acute hepatic disease was suggested by Jones and Eaton (6) who pointed out that the convalescent phase of acute hepatitis was frequently initiated by diuresis which they assumed was a result of the mobilization of previously stored water. Since reasonably reliable methods exist for the measurement of individual body fluid spaces and have been applied with success to the study of other infections (7), it was thought that a systematic study of the distribution of body water during the course of acute infectious hepatitis would reveal the presence of water storage and would further serve to identify the individual fluid compartments participating. In addition, periodic measurements of separate fluid shifts during the course of the disease would give quantitative expression to the relative role played by each fluid compartment while operating under hepatic control.

In a previous communication (8) data from one typical case were presented. However, in observing the fluid alterations in a group of 14 patients with acute infectious hepatitis, the movements of body water in each case were found to follow a pattern of great similarity. It was, therefore, thought that a presentation of consolidated data from the entire study would serve to emphasize the uniformity of these phenomena.

The present study was concerned with 14 young adult males who were part of a large group of Navy personnel with acute infectious hepatitis observed at the Hospital of The Rockefeller Institute for Medical Research (9). Each patient had been previously healthy and had developed hepatitis during active naval service. Only those cases in the early acute phase of hepatitis were included in the present study. Since water and electrolyte measurements were of primary interest, it was thought advisable to study only those patients who had been able to maintain a fluid intake approximating normal and in whom diarrhea, vomiting, severe fever, and dehydration were not complicating features of the acute phase of illness. The course of disease was comparable in each patient, being of moderate severity and proceeding in each instance through uncomplicated convalescence to complete clinical healing (10).

METHODS

On admission to the hospital, each patient was placed at bed rest in the Metabolic Ward where the entire study was conducted. The diagnosis of acute infectious hepatitis was readily established after a careful medical history and physical examination, and tests of liver function served to confirm the presence of acute liver disease in each instance. A urine examination done on admission and repeated frequently during the hospital course ruled out underlying renal disease in each case. Strict control of water balance was established by measurements of fluid intake and urine output and daily determinations of body weight. No patient received additional fluid by clysis. The entire group received a diet composed of 150 grams of protein, 50 grams of fat, and sufficient carbohydrate to maintain the daily food intake between 3,000 and 3,500 calories. The total daily salt intake including the calculated intrinsic food salt was kept constantly between 9 and 10 grams.

The initial measurements of plasma volume, blood volume, and "extravascular thiocyanate space" were begun under basal conditions on the day following admission to the hospital and repeated at least every 7 days, occasionally as often as every 4 days. The simultaneous method of Gregersen and Stewart (11), based upon the plasma dilution of the dye T-1824 and the "extravascular space" dilution of sodium thiocyanate, was adapted to the Coleman Jr. spectrophotometer. The selectivity of this instrument minimized the theoretical effect of the inter-

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ference of bilirubin and hemolysis (12) on the determination of T–1824. Dye-free samples of serum obtained before injection of T–1824 were used for control readings. Many preliminary tests on other patients with hepatitis indicated that 15 minutes was an appropriate interval for the "mixing time" of the dye in each case (13). All blood samples were obtained without the use of a tourniquet.

In the present study the "extravascular thiocyanate space" was derived by subtracting the plasma volume from the total thiocyanate space. So little change was encountered in the total red blood cell volume as expressed by the venous hematocrit (14) that small variations in the amount of red cell water were not considered of importance for these calculations (11). The total blood volume was calculated from simultaneous determinations of the plasma volume and venous hematocrit according to the formula (15):

$$\frac{\text{Plasma volume}}{(100-\text{hematocrit})}\times 100=\text{Blood volume.}$$

The measurements of all body fluid compartments were finally expressed on a per kilogram of body weight basis according to previously described standards (16). No measurements of the intracellular fluid phase were undertaken.

It had been previously determined (9) that the most reliable index of liver function during the course of acute infectious hepatitis is a combination of measurements of the total serum proteins with estimations of the albumin and globulin fractions, the plasma bilirubin level, the bromsulfalein exerction test (5 mgm. per kgm.—45-

TABLE 1

Alterations in body fluids, the components of blood and urine, and tests of liver function during acute infectious hepatitis

Case no.	Age	Day of disease	Day since onset dark urine	Body wt.	Hematocrit	Thiocyanate space	Plasma vol.	Blood vol.	Plasma proteins				NPN	Serum bilirubin	Bromsulfalein retention*	Thymol turbidity	Water tolerance test†	Chlorides		
									Total	Albumin	Globulin	A/G						Plasma	Urine	
				kgm.		*ml. per kgm. body wt.*	*ml. per kgm. body wt.*	*ml. per kgm. body wt.*	*grams per cent*				*mgm. per cent*	*mgm. per cent*	*per cent*	*units*		*m. eq. conc. per liter*	*m. eq. conc. per liter*	*m. eq. 24 hr. output*
1	32	7	2	80.4	51	200	42	85	6.42	3.30	3.12	1.1	21.2	6.6	43.0	23	794	95.8	27.8	114
		13	8	79.4	51	186	40	81	7.31	3.87	3.44	1.1	30.0	4.2	31.0	24	1,272	97.8	66.8	193
		20	15	78.6	47	175	37	70	7.11	3.90	3.21	1.2	36.2	1.9	16.5	19	1,296	99.8	55.8	167
		27	22	77.8	50	170	32	62	7.32	3.99	3.33	1.2	40.0	1.3	10.0	15	1,307	99.8	52.8	153
		34	29	78.0					7.36	4.28	3.08	1.4	35.0	1.0		11	1,446	101.0	97.8	169
2	17	6	3	56.2	48	218	56	107	6.54	4.26	2.28	1.9	28.7	4.0		13	1,183	96.8	56.8	165
		8	5	56.6	47	204	41	78	6.47	3.96	2.51	1.6	26.2	2.2	34.5	13		100.0	71.6	145
		15	12	58.1	45	201	44	80	7.86	4.76	3.10	1.6	28.7	0.7	10.5	12	1,305			
		22	19	59.3	44	197	38	67						0.5	11.5	6	1,572			
3	19	4	3	78.3	51	169	45	91	6.10	3.40	2.70	1.3	36.2	6.9	45.0	17	519	93.0	18.8	42
		11	10	77.7	51	148	44	90	7.10	3.64	3.46	1.1	37.5	1.6	4.5	30	1,037	95.8	61.8	173
		18	17	76.8	52	148	50	104	7.20	4.45	2.75	1.6	35.0	1.3	7.5	20	1,459	99.8	93.0	273
		25	24	77.0	50	149	50	99	6.65	4.22	2.43	1.7	35.0	0.4	2.5	21	1,726	102.0	82.0	225
		32	31	77.8	49	151	49	96	8.01	4.94	3.07	1.6	30.0	0.8	1.0	14	1,817	102.0	128.0	183
		49	48	77.7	49	155	47	92	6.49	4.10	2.39	1.7	23.7	0.4	1.2	8		101.0	142.0	216
4	25	8	4	75.7	51	190	52	107	5.61	3.56	2.05	1.7	25.0	4.9	39.5	20	439	93.8	36.8	79
		15	11	75.3	51	185	40	82	6.35	3.67	2.68	1.4	32.5	1.6	3.8	19	947	98.8	52.0	126
		23	19	76.3	49	170	45	88	6.66	4.61	2.05	2.2		0.8	0.5	12	1,218	99.8	76.0	130
		30	26	77.6	47	162	45	85	6.94	4.77	2.17	2.2	26.2	0.6	0.5	9	1,618	101.0	80.0	173
		47	43	79.1	50	170	41	82	6.55	4.03	2.52	1.6	26.2	0.6	0.5	8				
5	20	7	4	63.5	50	258	43	86	5.91	3.08	2.83	1.1	26.3	9.7	39.5	25	1,251	99.0	53.0	171
		15	12	64.7	49	234	43	83	6.81	4.36	2.45	1.8	22.5	2.8	21.5	29	1,248	103.0	96.0	241
		22	19	65.0	48	216	41	79	7.59	4.34	3.25	1.3	35.0	1.6	21.5	20	1,205	100.8	82.0	162
		29	26	66.0	48	210	38	73	7.25	4.69	2.56	1.8	38.7	1.0	10.0	16	1,344	101.0	73.0	148
		36	33	67.4	47	202	36	68	7.46	5.06	2.40	2.1	30.0	0.8	4.0	16	1,221	101.0	101.0	172
		51	48	66.9					6.56	4.40	2.16	2.0	25.0	0.5	0.0	12	1,222			
6	17	7	6	66.2	49	182	58	113	6.73	4.34	2.39	1.8	35.0	2.9	29.5	11	1,167	95.0	54.0	136
		15	14	66.3	49	170	44	86	6.81	4.47	2.34	1.5	35.0	1.4	23.0	9	1,329	98.8	109.0	167
		23	22	67.6	50	176	40	81	7.24	4.70	2.54	1.6	27.5	1.2	8.5	7	1,210	101.0	113.0	213
		30	29	69.4	48	174	38	73	7.17	4.71	2.46	1.9	26.2	0.8	3.7	4	1,153	101.0	96.8	177
		37	36	70.5	47	183	39	73	7.03	4.84	2.19	2.2	25.0	0.7	2.5	4	1,117	101.0	116.0	188
		53	52	72.1	47	180	40	74	6.45	4.25	2.20	1.9	30.0	0.5	2.5	2	1,302			
7	19	10	6	59.5	46	198	49	92	6.58	3.91	2.67	1.5	33.7	7.1	50.5	14	392	98.2	48.2	66
		18	14	60.1	46	189	52	95	6.73	3.89	2.84	1.4		1.6	3.5	7	1,584	99.8	74.0	186
		25	21	60.8	46	181	57	106	6.40	4.21	2.19	1.9		1.0	4.5	5	1,709		113.0	241
		31	27	62.1	46	183	45	83	6.76	4.64	2.12	2.0	18.7	0.7	2.0	8	1,524	102.0	95.0	214
		38	34	63.4	46	185	41	75	6.78	4.83	1.95	2.5	26.2	0.9	0.5	4	1,698	100.0	111.0	276
		52	48	63.0	45	186	47	85	7.29	4.54	2.75	1.7	32.5	0.8	1.2	4	1,536	103.0	137.0	218
8	21	7	6	49.6	48	245	43	83	7.60	4.04	3.56	1.1	32.5	7.3	41.0	13	775	95.0	46.0	147
		10	9	49.5	48	239	42	80	6.82	4.23	2.59	1.6	32.5	8.3		20	1,346	94.0	80.0	163
		18	17	49.6	47	216	43	80	7.36	3.98	3.38	1.2	35.0	1.8	7.5	14	1,341	102.0	110.0	223
		25	24	51.5	48	215	35	67	7.78	4.56	3.22	1.4	30.0	0.8	4.5	12	1,343	103.0	89.0	165
		33	32	52.6	46	205	36	67	7.64	4.71	2.93	1.6	26.3	1.0	7.5	7		101.0		
		45	44	53.4	46	217	36	66	7.32	4.70	2.62	1.8	27.5	0.7	1.0	4	1,326	102.0		

TABLE I—*Continued*

Case no.	Age	Day of disease	Day since onset dark urine	Body wt.	Hematocrit	Thiocyanate space	Plasma vol.	Blood vol.	Plasma proteins: Total	Plasma proteins: Albumin	Plasma proteins: Globulin	Plasma proteins: A/G	NPN	Serum bilirubin	Bromsulfalein retention*	Thymol turbidity	Water tolerance test†	Chlorides: Plasma	Chlorides: Urine	Chlorides: Urine
				kgm.		*ml. per kgm. body wt.*	*ml. per kgm. body wt.*	*ml. per kgm. body wt.*	*grams per cent*				*mgm. per cent*	*mgm. per cent*	*per cent*	*units*		*m. eq. conc. per liter*	*m. eq. conc. per liter*	*m. eq. 24 hr. output*
9	25	15	6	81.4	46	218	45	83	6.81	3.99	2.82	1.4	21.2	5.3	38.5	23	1,282	96.8	50.0	129
		24	15	80.0	44	191	·41	73	7.32	4.07	3.25	1.3	28.7	1.4	5.0	12	1,451	101.0	92.0	135
		31	22	79.6	45	193	40	72	6.98	4.17	2.81	1.5	32.5	1.0	3.5	13	1,483	103.0	82.0	125
		38	29	79.4	48	195	40	76	7.61	4.36	3.25	1.3	31.2	0.9	2.0	12	1,545	104.0	87.8	98
		45	36	79.6	47	192	40	76	6.91	4.57	2.34	1.9	28.7	0.7	2.0	8	1,548	107.0		
		50	41	79.8					6.23	4.26	1.97	2.2	27.5	0.5	2.0	9	1,498			
10	34	12	7	56.6	46	242	49	90	6.25	3.97	2.28	1.7	25.0	5.0	35.5	21	503	97.0	17.0	55
		16	11	56.0	46	224	46	85	5.85	4.09	1.76	2.3	25.0	5.0	33.5	21		102.0	41.0	92
		23	18	56.6	45	223	45	82	6.23	4.07	2.16	1.9	28.7	1.9	8.0	17	1,170	101.0	27.0	116
		30	25	57.1	45	216	40	72	6.24	4.21	2.03	2.1	26.2	0.9	10.0	13	1,358	103.0	47.0	144
		36	31	57.0	47	217	40	75	7.33	4.83	2.50	1.5	26.3	1.0	5.0	14	1,341	102.0	45.0	134
		51	46	57.4	47	219	41	77	6.85	4.70	2.15	2.2	28.7	0.6	6.0	10	1,529	103.0		
11	22	10	10	85.8	51	200	41	83	5.79	3.55	2.24	1.6	21.2	7.8	43.0	16	601	90.8	27.8	55
		20	20	83.8	50	187	43	85	6.91	3.94	2.97	1.3	18.7	3.4	11.0	21	1,520	96.0	107.0	171
		26	26	84.0	49	182	38	74	7.46	4.25	3.21	1.3	30.0	1.9	10.0	15	1,367	99.0	139.0	200
		33	33	82.8	47	166	39	73	7.06	4.43	2.63	1.7	31.2	1.3	4.5	10	1,407	99.0	103.0	132
		37	37	82.4	48	163	36	69	6.51	4.54	1.97	2.3	32.5	1.2	2.5	10	1,391	103.0	139.0	204
12	23	17	10	57.3	47	194	45	84	7.23	3.75	3.48	1.1	30.0	2.8	23.0	17	1,376	98.3	83.0	111
		27	20	57.7	47		47	89	6.90	4.45	2.45	1.8		1.4	5.0	15	1,409	103.0	110.0	254
		33	26	57.0	46	193	54	100	6.97	4.40	2.57	1.7		1.3	5.0	12	1,677	101.0	96.0	230
		40	33	58.5	46		49	91	6.93	4.59	2.34	2.0	16.4	0.8	2.0	9	1,506	102.0	123.0	262
		47	40	59.7	47	198	40	75	6.89	4.86	2.03	2.4	21.2	0.7	2.5	6	1,367	100.0	139.0	261
		59	52	61.3	51	192	40	81	7.31	5.31	2.00	2.7	30.0	0.6	2.0	4	1,484	102.0	141.0	266
13	21	12	12	61.0	48	184	55	106	6.63	3.83	2.80	1.4	26.2	8.9	37.5	8	725	97.0	55.6	151
		18	18	61.2	48	175	46	88	6.61	3.69	2.92	1.3		2.9	18.0	8	1,542	98.0	78.8	248
		25	25	61.6	46	171	59	91	6.71	4.39	2.32	1.5	37.5	1.6	2.0	8	1,710	105.0	87.0	208
		33	33	62.4	44	171	54	97	7.39	4.95	2.44	2.0		1.2	1.2	5	1,398	104.0	88.0	210
		42	42	63.7	49	172	44	86	7.45	4.87	2.58	1.9	17.5	0.7	2.0	6	1,384	102.0	101.0	213
		60	60	65.1	49	173	40	79						0.6	0.5	7		102.0	100.0	206
14	53	19	14	80.1	44	196	49	87	6.21	2.80	3.41	0.8	31.2	23.2	52.0	37	612	98.2		
		27	22	77.9	43		43	76	6.11	2.79	3.32	0.8	32.5	18.2	49.0	30	522	99.1	21.0	56
		35	30	75.6	41	198	46	78	6.07	3.24	2.83	1.1		13.0	39.5	28	752	101.0	30.8	78
		41	36	74.9	41		53	91	6.03	3.53	2.50	1.4	35.0	6.9	27.5	27	703	104.0	82.0	236
		47	42	75.6	40	201	46	76	5.94	3.33	2.61	1.3	21.2	4.6	16.0	19	1,010	103.0	92.0	225
		55	50	75.5	41	188	45	77	6.77	4.01	2.76	1.5	27.5	3.4	17.0	17	1,048	101.0	103.0	215
		62	57	76.1	41	186	41	69	6.38	3.70	2.68	1.4	28.7	2.5	10.0	11		104.0	75.0	237
		69	64	77.8	41	189	39	66	5.52	3.71	1.81	2.0	27.5	1.7	10.5	11		102.0	50.0	157
		76	71	77.8	43	182	44	78	6.67	3.86	2.81	1.4	31.2	1.4	7.0	9	1,062	103.0	56.8	168
		83	78	78.2	44	183	36	65	6.54	3.95	2.59	1.5	27.5	1.0	6.5	9	1,101	101.2	67.0	163

* 5 mgm. per kgm. of body weight; 45-minute interval.
† Urine volume excreted during 4-hour period following ingestion of 1,500 ml. of water under basal conditions.

minute specimen), and the thymol turbidity reaction of the serum. These measurements and tests were performed each week and correlated with the above described body fluid measurements (Table I and Figure 3). The total plasma protein and albumin and globulin fractions were determined by a salting out procedure in which micro-digestion and Nesslerization were used (17). The plasma bilirubin method of Malloy and Evelyn (18) was adapted to the Coleman Jr. spectrophotometer, as was the bromsulfalein test (19) and the thymol turbidity reaction of the serum (20, 21). Blood chlorides were measured according to the methods of Wilson and Ball (22) on blood samples collected under oil. The same method was also applied to urine and all values were expressed as milliequivalents of chloride per liter.

In an attempt to reveal water storage and to give quantitative expression to the tendency for fluid retention, the patients under study were periodically "challenged" with a water tolerance test (23). Under basal conditions the patients drank 1,500 ml. of tap water in a 20-minute period or less and the volume of urine excreted every half hour for the first hour and every hour thereafter for a total period of 4 hours was recorded. These tests were not undertaken on the days that the body fluid compartments were measured.

RESULTS

In Table I are shown the correlated data obtained in each case during simultaneous observations on body fluids, water tolerance, tests of liver function and measurements of the hematocrit, plasma proteins, and chlorides at periodic intervals in the course of acute hepatitis.

It is apparent that all patients exhibited greater thiocyanate spaces during the acute phase of hepatitis than later in the course of convalescence. The absolute and percentile increase in each case may

be seen by reference to Table II. Although great individual variation is evident in the degree of change seen, the average expansion of this compartment over the lowest convalescent level was 24.5 ml. per kgm. of body weight which represents a 13.5 per cent increase. With the exception of case 3, the plasma and blood volumes were also expanded during this period, less in absolute amount than the thiocyanate space, but greater when considered in terms of per cent of the lowest convalescent values. Although the average increase in plasma volume was 9.3 ml. per kgm. of body weight, and the same value for blood volume was 18.9 ml. per kgm., the per cent increase in these two compartments was 23.4 per cent and 27.1 per cent, respectively. Since these two figures indicate relatively identical percentage enlargements, it is evident that expansion of both the plasma and blood volumes during the acute phase of hepatitis occurs in a symmetrical manner. The increase in total circulating red cell volume, therefore, must keep pace with the swelling plasma volume, resulting in minimal alterations in the venous hematocrit throughout the course of illness. The acute phase of infectious hepatitis in comparison to convalescence is, therefore, characterized by an increase in both total red cell volume and plasma volume [polycythemic hypervolemia (38)].

TABLE II

Tabular listing of 14 cases of acute infectious hepatitis to illustrate the absolute and percentile expansion of the thiocyanate space, plasma volume, and blood volume during the acute phase, with the lowest convalescent values as a base line

Case number	Increase in thiocyanate space		Increase in plasma volume		Increase in blood volume	
		Increase		Increase		Increase
	ml. per kgm.	*per cent*	*ml. per kgm.*	*per cent*	*ml. per kgm.*	*per cent*
1	30	17.7	10	33.0	23	37.1
2	21	10.7	18	48.0	40	59.8
3	21	14.2	−5*	−10.0*	−13*	−12.5*
4	28	16.5	12	29.3	25	30.6
5	56	27.7	7	19.2	18	26.6
6	12	7.1	20	52.6	40	54.8
7	17	10.7	8	9.5	17	22.7
8	40	19.5	8	22.9	17	25.7
9	27	14.1	5	12.8	7	9.2
10	25	11.4	9	22.5	18	25.0
11	37	22.7	5	13.9	14	20.2
12	2	1.0	5	12.7	9	12.0
13	13	7.6	15	37.2	27	34.2
14	14	7.7	13	36.1	22	34.1
Average increase	24.5	13.5	9.3	23.4	18.9	27.1

* Decrease.

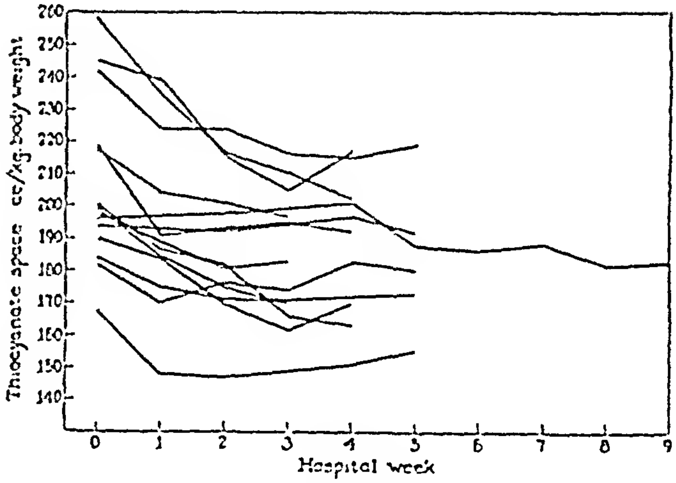

FIG. 1. ALTERATIONS IN THIOCYANATE SPACE IN 14 PATIENTS DURING THE COURSE OF ACUTE INFECTIOUS HEPATITIS

The rate of fall in the individual fluid compartments with recovery may be seen by reference to the individual columns in Table I. The composite values for the thiocyanate space throughout the course of illness are graphically represented in Figure 1. The rate of fall in this compartment was greatest during the first week of hospitalization and was usually detectable by a loss in body weight quite comparable to the amount of fluid lost by reduction in the size of this compartment. When mobilization of this fluid space occurred rapidly, a detectable diuresis appeared (Figure 3). Despite the demonstration of an expanded thiocyanate compartment in every case studied, edema and ascites were never clinically apparent.

The variable urine volume following ingested water during these periods was of particular interest. The water tolerance test previously described yielded progressively larger excretory volumes during the course of convalescence. The greatest increase in the output of urine following the 1,500 ml. drink of water occurred when the thiocyanate space was maximally contracting and was thought to indicate a decreasing tendency to retain water. The excretion patterns obtained at different periods of illness were quite distinct. An example of delayed urine output following the test dose of water given during the most acute phase of illness is illustrated in the slowly rising pattern of Figure 2A (case 10). In this instance

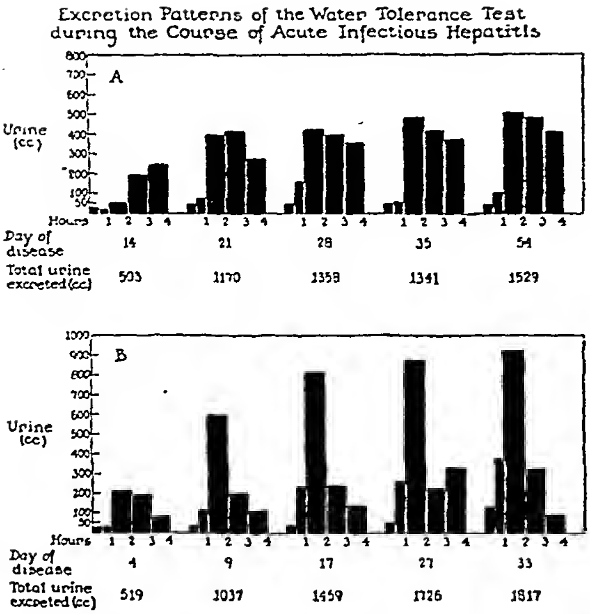

FIG. 2. EXCRETION PATTERNS OF THE WATER TOLERANCE TEST DURING THE COURSE OF ACUTE INFECTIOUS HEPATITIS

there was a step-wise increase in urine volume over the 4-hour period of observation. Later in convalescence larger amounts of urine were excreted in the earlier time periods of the test and a "shift to the left" occurred in the pattern (23). The total urine volume of the test period likewise increased with convalescence. The peak of excretion during the recovery period was usually reached in the second hour of the test. In many cases these peaks were conspicuous (Figure 2B, Case 3) and increased in size as the patient recovered. With the adjustment of the thiocyanate space to base line convalescent levels, little tendency to retain ingested water was noted as measured by the water tolerance test and almost the entire volume of ingested water was excreted within the 4-hour period and often surpassed.

Tests of liver function applied during the observations of fluid shift indicated progressive restoration of function. The extent of hepatic control over the dynamics of this shifting fluid pattern may be appreciated from the fact that the largest movements in body fluid occurred during those periods of greatest improvement in hepatic function and were most apparent during the first two weeks of hospitalization. Following this period, both hepatic function and fluid shift moved less rapidly towards stabilization.

A slight depression of plasma protein concentration was observed during the acute phase of illness that was undoubtedly due in part to the hypervolemia and in part to nutritional factors. These changes were of too little magnitude to have been influential in affecting the fluid movements described above. However, a secondary rise in plasma volume was noted in cases 2, 3, 7, 12, 13 and 14 during the second week of recovery which often approached the level of the plasma volume obtained during the early acute phase of the disease. This may have been due to the slight but inconstant rise in the total circulating albumin which occurred at this time, possibly on the basis of enhanced nutrition and improvement in albumin synthesis by the liver.

The behavior of the plasma and urine chlorides during the course of acute hepatitis was thought to be of importance because of the previous demonstration of chloride retention in cirrhosis and liver atrophy (24, 25), sodium retention in "catarrhal" jaundice (26), and disturbance in the metabolism of inorganic salts of the blood and tissue in various hepatic diseases (27). In addition, it had been shown that depressed plasma and urine chloride levels develop in animals following acute liver injury by arsphenamine (28).

On admission, the majority of patients revealed low plasma chlorides varying from 90 to 98 meq. per liter. The urine chloride concentration and total 24-hour urine chloride excretion were similarly depressed at this early period. With recovery and mobilization of the expanded thiocyanate space, the plasma chlorides rose and increasing amounts of chloride appeared in the urine. This rise could not be explained wholly by the chloride intake of the patient which was kept constantly between 9 and 10 grams daily, because it often occurred several days after the beginning of the constant salt intake. More often these adjustments awaited the contraction of the thiocyanate fluid compartment. In the presence of an increased thiocyanate compartment, as well as an increased plasma and blood volume, it was difficult to attribute the low urine volume, depressed urine chlorides, and tendency for water storage to simple dehydration (29). It was believed on the basis of these observations, therefore, that increased amounts of chloride were held in the expanded thiocyanate compartment during the acute stage of

hepatitis, in compliance with the laws of diffusion equilibria, and that with contraction of this compartment during recovery, increased quantities of chloride were mobilized and appeared in greater amounts in both plasma and urine.

Presentation of data from case 10 (Figure 3) with a description of the course of the disease will serve to correlate more clearly the movements of body fluids, the tendency to store fluid, the alterations in blood constituents, and the state of liver function during the course of acute infectious hepatitis.

F. J. E., a 34-year-old naval lieutenant commander, was admitted to the Hospital of The Rockefeller Institute on March 24, 1946, complaining of jaundice. Twelve days before admission he had developed a mild headache followed by anorexia, malaise, generalized muscular aches, and lassitude. He went to bed and felt slightly better after 3 days of rest, fluids, and a moderate food intake. During this time light tan stools were passed and 2 days later darkening of the urine appeared. By March 22, ic-

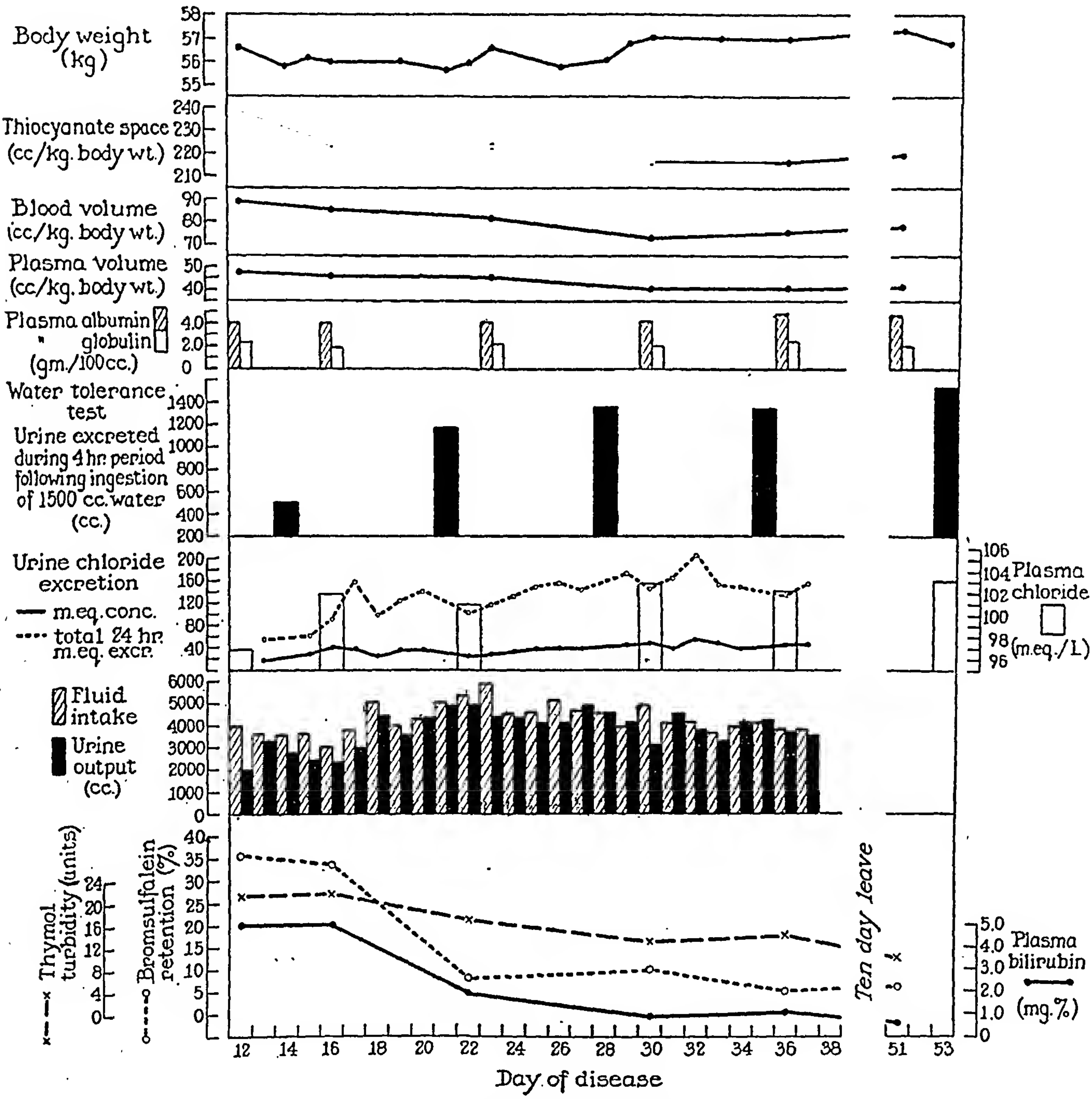

FIG. 3. THE DATA FROM CASE 10 CORRELATING THE ALTERATIONS OF THE BODY FLUID COMPARTMENTS, WATER TOLERANCE, BLOOD CONSTITUENTS, CHLORIDES AND WATER BALANCE WITH RECOVERY OF LIVER FUNCTION DURING THE COURSE OF ACUTE INFECTIOUS HEPATITIS

terus of the sclerae was noted but he was better able to eat and maintain his fluid intake. There was no history of previous illness, transfusions, inoculations or exposure to toxic chemical agents. One month before onset, however, the patient's fiancée had developed acute infectious hepatitis and was moderately ill for a period of 10 days. Physical examination of the patient on admission revealed a lean, muscular, adult male with a rectal temperature of 99.2° F.; he appeared to be moderately ill. There was marked icterus of the skin and sclerae. No rash or spider angiomata was discernible. The edge of the liver was felt 1.0 cm. below the costal margin and was tender; the tip of the spleen descended one finger's breadth below the left costal margin. There was no apparent edema or ascites. The remainder of the physical examination was negative.

Laboratory data on admission revealed a hematocrit of 46, normal blood counts, a negative urine examination, urine specific gravity 1.010, serum bilirubin of 5 mgm. per cent, bromsulfalein retention of 35.5 per cent, and a thymol turbidity reaction of 21.3 units. The total plasma proteins were 6.25 grams per cent, albumin 3.97 grams per cent and globulin 2.28 grams per cent with an A/G ratio of 1.7.

The patient was given the diet and salt intake previously prescribed for this group and careful measurements of fluid intake and urine output were begun.

The patient was completely afebrile while in the hospital. Following the institution of bed rest and adequate nutrition, recovery proceeded without complication. The urine became lighter, increased color appeared in the stool, icterus diminished and disappeared. The liver and spleen receded in size, finally becoming impalpable.

The initial measurements of the body fluid compartments were undertaken the day following admission. The first 2 hospital days were marked by a fall in body weight amounting to 0.8 kilogram. Parallel with this, the thiocyanate space contracted from 237 ml. per kgm. of body weight to 223 ml. per kgm. of body weight. This represented a contraction of 14 ml. per kgm. of body weight or a total of 784 ml. which could quite accurately account for the drop in body weight described above if this fluid volume was simultaneously excreted. A slight decline in blood and plasma volume accompanied these changes. During convalescence there was marked improvement in the tests of liver function and the final value for the thiocyanate space was 215 ml. per kgm. after recovery was complete.

In spite of a fluid intake of 3,000 ml. to 4,000 ml. from the time of admission, the urine volume remained small. In addition, the plasma and urine chloride concentrations remained low. The water tolerance test, performed on the third hospital day, yielded only 503 ml. of urine following the ingestion of 1,500 ml. of tap water. By the 17th day of illness, when marked improvement in liver function was evident, there appeared an increase in urine output which approximated 85 per cent to 90 per cent of the fluid intake for any given day. Although the salt intake had been maintained between 9 and 10 grams since admission, chlorides did not appear in the urine in increased amounts until this period of diuresis. On the 21st day of illness, 1,170 ml., or more than double the previous urine volume, was excreted during the water tolerance test. Subsequent tests showed slight increase over this value (Figure 2A). For the remainder of the period of study the daily urine volume remained at normal levels. A weight gain appeared in the mid-period of recovery which was probably a result of improvement in nutrition and liver function. By the time of discharge from the hospital the gain in body weight had been held, liver function had been adequately restored, and recovery was considered complete. In addition, a 10-day leave period had been tolerated without difficulty. The minimal fluctuations in plasma protein concentration noted during the course of illness bore no constant relation to the alterations of body fluids described above. Throughout the period of hospitalization, dehydration, edema, and ascites had never become clinically evident.

DISCUSSION

The expanded thiocyanate compartments and the relatively poor water tolerance of patients during the acute phase of infectious hepatitis substantiate the concept of Jones and Eaton (6) that increased water storage occurs during this period of the disease. It was also their suggestion that the diuresis of convalescence resulted from improved hepatic efficiency, and that this resulted in a "shift of fluid from the tissues or serous cavities to the blood stream with the ultimate establishment of diuresis." In keeping with these suggestions, the shifts of body fluid, as demonstrated by the present study with methods that offer quantitation of individual fluid compartments, reveal shrinkage of the thiocyanate compartment during recovery with an accompanying rise in urine output. The tendency to retain water during acute hepatic in-

sufficiency, as expressed by the water tolerance test, has been noted to occur in infectious hepatitis (23) as well as in acute liver injury produced by chemical substances (28, 30). In the partially hepatectomized dog, hydrothorax may develop within a few days of operation, often of sufficient amount to drown the animal (31). Ascites may also appear despite the presence of an Eck fistula. In addition, total extirpation of the liver in frogs has been shown to result in tremendous edema (32) and decreased water tolerance (33). The oliguria and anuria of yellow fever are reported to be most severe in those cases in which there is a correspondingly great amount of hepatic necrosis (34). It has been suggested that the increased retention of water resulting in hyperhydration of the body because of damage to organs such as the liver might serve a useful purpose in diluting and overcoming the harmful effects of too great a concentration of injurious substances formed during infection (35). Similar water retention phenomena have been produced by dietary liver injury as demonstrated by the experiment in which rats kept on a high fat diet when dehydrated retained more from a given quantity of water than did normally fed animals under the same conditions (36).

The expansion of the thiocyanate space during the acute phase of hepatitis, together with the enlarged plasma volume, signifies a general distension of all the extravascular spaces. The minimal change in the venous hematocrit noted throughout the course of hepatitis, despite the increased plasma volume, must indicate a symmetrical enlargement of the total red cell volume. However, the slight reticulocytosis during this period of the disease could not account for the increase in the red blood cell volume on the basis of the formation of new red cells, although extramedullary hematopoiesis has been reported in fatal cases of acute liver disease (37). A partial explanation may reside in the fact that the acutely inflamed liver fails to store blood efficiently with the result that more blood remains in the general circulation (38, 39). During the acute stage of hepatitis, wide-spread edema and necrosis occupy much of the liver substance (40, 41) which, under these circumstances, probably contains relatively less blood. In addition, sinusoidal congestion is more evident during the healing phase of hepatis. Although there is no information available relating to the hepatic blood flow and total blood contained within the liver during acute infectious hepatitis, it is not unreasonable to consider that acute inflammatory disease of the liver might shunt large amounts of blood to the general circulation. Simple hypervolemia has been reported in cirrhosis of the liver (42, 43).

A partial explanation for the maintenance of the hematocrit in the face of an expanded plasma volume might be found in the slight macrocytosis of acute hepatitis. Variations in the Mean Corpuscular Volume of from 95 cu. μ to 120 cu. μ and in the Mean Corpuscular Hemoglobin Concentration of from 28 per cent to 35 per cent, have been encountered in hepatitis (44, 45), suggesting that the red cells may be swollen during the acute phase of jaundice but gradually return to normal size with recovery of the patient.

The results of the investigations cited above have served to indicate in outline the conditions under which water retention may occur in the presence of diminished liver function, and have pointed out the possible factors believed to be responsible for the expansion of the plasma and blood volumes during acute hepatitis. There remain to be defined the forces underlying this shifting fluid pattern.

The exact role of the kidney in relation to these movements of fluid in liver disease has never been established. While the "hepato-renal" syndrome clinically suggests that disastrous disturbances in water balance may occur under combined hepatic and renal control, biochemical and metabolic studies of this state have failed to disclose what fundamental mechanisms are aberrant (46, 47).

The fluid content of the tissues has been considered to be under hepatic control by virtue of metabolic (48) and hormonal regulation (49, 50, 51). Relevant to this concept are the unconfirmed data indicating that the acute phase of hepatitis is accompanied by an increase in the urinary excretion of biologically active estrogen (52). The water-storing influence of active estrogen is well recognized and is commonly encountered in the premenstrual state when there may occur edema, swelling of the breasts, and a gain in body weight (53).

A recent report (54) indicated that the urine of patients with cirrhosis and ascites, when injected into hydrated rats, delayed the excretion of urine.

When the urine of cirrhotic patients in whom ascites had been controlled was injected, it was found to have antidiuretic activity greater than normal urine but less than urine from ascitic patients. In 6 patients with cirrhosis, re-accumulation of ascitic fluid ceased with dietary therapy and intravenous liver extract before demonstrable increase in the plasma albumin levels. This experience has been confirmed (55) and emphasizes the importance of factors other than the serum proteins that may have influence over water storage during liver disease. This has been a matter of comment in reference to the general problem of edema for many years (56).

The serum protein concentration and total circulating protein exhibited little alteration during the course of acute hepatitis in the 14 patients in the present study and bore no constant relation to the changes in plasma volume. The quantitative changes in the plasma proteins that may occur independent of alterations in the plasma volume have been reported experimentally and are to be found especially where nutritional features are of great importance (57). The results of the present work, therefore, suggest that factors other than the simple effect of the serum proteins account for water storage and decreased water tolerance during the acute phase of jaundice and the movement of water from the interstitial fluid spaces with recovery. Reference to the antidiuretic principle, therefore, seems to be pertinent. The pituitary origin of this substance has been suggested (58, 59) but requires further elucidation. Heller and Urban (60) have demonstrated that normal liver *in vitro* possesses great capacity to adsorb (inactivate) the antidiuretic principle of the posterior lobe of the pituitary when compared with blood, muscle, brain and kidney. Boiled liver tissue, however, was completely ineffective. In addition, following the intravenous injection of large doses of pituitrin, there was rapid disappearance of antidiuretic activity from the circulating blood. These experimental data suggest that the normal liver inactivates the antidiuretic principle and thereby maintains normal diuresis and body water content. In acute hepatitis it is conceivable, therefore, that with diffuse hepatic damage and depression of liver function, this inactivation is incompletely performed and water retention results. With recovery and improvement in hepatic efficiency, inactivation of the antidiuretic principle is resumed, resulting in diuresis and shrinkage of the thiocyanate space to a normal volume. Bioassays of the antidiuretic substance in the urines of patients with acute infectious hepatitis are now under way in this laboratory.

SUMMARY

1. Periodic observations of the thiocyanate space, total blood and plasma volumes were undertaken during the hospital course of 14 patients with acute infectious hepatitis under conditions of rigid water balance and correlated with measurements of plasma and urine chlorides, the tendency to retain ingested water as measured by a water tolerance test, and tests of liver function.

2. During the acute phase of hepatitis, the thiocyanate compartment and blood and plasma volumes were found to be expanded. Depressed plasma and urinary chlorides as well as an increased tendency to retain ingested water accompanied these changes.

3. With convalescence and improving liver function, shrinkage of the thiocyanate compartment and a decrease in the total blood and plasma volumes occurred. A diuresis and decreased tendency to retain ingested water were noted at this time and a rise in plasma and urinary chloride levels followed.

4. The slight alterations in total circulating protein were thought to be attributable to nutritional factors and to decreased protein synthesis during hepatitis and were not considered to be of major importance in determining these fluid shifts.

5. It is suggested that these movements in body fluids during acute infectious hepatitis may be directed by endocrine factors and related to incomplete inactivation of the antidiuretic principle by a damaged liver.[2]

[2] Recent studies by Shorr, Zweifach and Furchgott (Science, 1945, 102, 489) have indicated that a vasodepressor substance of hepatic origin (VDM) is associated with the opening up of the capillary bed during the decompensatory stage of experimental shock. More recently, in studies with Furth and Sobol, they have also been able to detect VDM in the blood of mice with granulosa cell tumors, concurrent with the development of hypervolemia in these animals (Science, 1947, 105, 41). VDM was also shown by them to appear in the blood of human beings with liver cirrhosis during periods of hepatic decompensation and in the blood and liver mash of rats with experimental hepatic cirrhosis. Finally, a

The advice and guidance of Dr. D. D. Van Slyke is gratefully acknowledged.

BIBLIOGRAPHY

1. Gilbert, A., and Lereboullet, P., Des urines retardées (opsiurie) dans les cirrhoses. Compt. rend. Soc. Biol., 1901, **53**, 276.
2. Van Noorden, C., Metabolism and practical medicine. Pathology, 1907, **2**, 267.
3. Steinthal, Ueber Icterus Gravis und Anure. Beitr. klin. Chir., 1911, **76**, 629.
4. Whitla, W., Uremia in affections of the liver. Dublin J. Med. Sci., 1876, **61**, 107.
5. Adler, A., Liver and diuresis. Klin. Woch., 1923, **2**, 1980.
6. Jones, C. M., and Eaton, F. B., The prognostic significance of a spontaneous diuresis in acute or subacute disease of the liver. New England J. Med., 1935, **213**, 907.
7. Rutstein, D. D., Thomson, K. J., Tolmach, D. M., Walker, W. H., and Floody, R. J., Plasma volume and "extravascular thiocyanate space" in pneumococcus pneumonia. J. Clin. Invest., 1945, **24**, 11.
8. Labby, D. H., and Hoagland, C. L., Alterations in the body fluids during acute infectious hepatitis. Proc. Soc. Exper. Biol. and Med., 1946, **63**, 110.
9. Hoagland, C. L., and Shank, R. E., Infectious hepatitis; Review of 200 cases. J. A. M. A., 1946, **130**, 615.
10. Kunkel, H. G., Labby, D. H., and Hoagland, C. L., Chronic liver disease following infectious hepatitis. I. Abnormal convalescence from initial attack. Ann. Int. Med. (In press.)
11. Gregersen, M. I., and Stewart, J. D., Simultaneous determination of the plasma volume with T-1824 and the "available fluid" volume with sodium thiocyanate. Am. J. Physiol., 1939, **125**, 142.
12. Phillips, R. A., A method for the determination of the blue dye T-1824 in plasma. J. Exper. Med., 1943, **77**, 421.
13. Gibson, J. G., Jr., and Evans, W. A., Jr., Clinical studies of blood volume; clinical application of method employing azo dye "Evans blue" and spectrophotometer. J. Clin. Invest., 1937, **16**, 301.
14. Wintrobe, M. W., Clinical Hematology. Lea and Febiger, Philadelphia, 1942.
15. Keith, N. M., Rowntree, L. G., and Geraghty, J. T., A method for the determination of plasma and blood volume. Arch. Int. Med., 1915, **16**, 547.

highly purified VDM concentrate, prepared from beef liver by Mazur in their laboratory, was found to exert a profound anti-diuretic effect in the normal dog on intravenous administration. These observations have led these investigators to suggest the causal relation between the hepatic vaso-depressor, VDM, and the increased blood volume and the anti-diuresis of hepatic cirrhosis and infectious hepatitis. (Personal communication.)

16. Gibson, J. G., 2nd, and Evans, W. A., Jr., Clinical studies of the blood volume. II. The relation of plasma and total blood volume to venous pressure, blood velocity, rate, physical measurements, age and sex in ninety normal humans. J. Clin. Invest., 1937, **16**, 317.
17. Archibald, R. M., Army Medical Laboratory Manual, 1947. To be published.
18. Malloy, H. T., and Evelyn, K. A., The determination of bilirubin with the photoelectric colorimeter. J. Biol. Chem., 1937, **119**, 481.
19. Mateer, J. G., Baltz, J. I., Marion, D. F., and MacMillan, J. M., Liver function tests., J. A. M. A., 1943, **121**, 723.
20. Shank, R. E., and Hoagland, C. L., A modified method for the quantitative determination of the thymol turbidity reaction of serum. J. Biol. Chem., 1946, **162**, 133.
21. Kunkel, H. G., and Hoagland, C. L., Persistence of elevated values for the thymol turbidity test following infectious hepatitis. Proc. Soc. Exper. Biol. and Med., 1946, **62**, 258.
22. Wilson, D. W., and Ball, E. G., A study of the estimation of chloride in blood and serum. J. Biol. Chem., 1928, **79**, 221.
23. Aldersberg, D., and Fox, C. L., Changes of the water tolerance test in hepatic disease. Ann. Int. Med., 1943, **19**, 642.
24. Kriss, B., and Pollak, L., Ueber die Chlorausscheidung bei Leberkranken nach Belastung mit Verschiedenen Chloriden. Wien. klin. Wchnschr., 1927, **40**, 1251.
25. Thiers, H., A propos des syndromes chloropéniques. Les chloropénies sans azotémie et les chloropénies cirrhotiques. État du chlore tissulaire. J. de Physiol. et de Path. Gén., 1930, **28**, 86.
26. Siedek, H., and Zuckerkandl, F., Der Natrium-Chlor Quotient bei Icterus Catarrhalis. Klin. Wchnschr., 1935, **14**, 568.
27. Shigemi, H., Studies on metabolism of inorganic salts and water in hepatic disturbances. Jap. J. Gastroenterol., 1935, **7**, 12.
28. Soffer, L. J., Dantes, A. D., and Sobotka, H., Electrolytes of blood and urine of dogs with acute hepatic injury produced by arsphenamine. Arch. Int. Med., 1937, **60**, 509.
29. Lyons, R. H., Jacobson, S. D., and Avery, N. L., Jr., The effect on the plasma volume of dehydration produced by a low salt diet and ammonium chloride. Am. Heart J., 1944, **27**, 353.
30. Aldersberg, D., and Paul, B., Ueber Veraenderungen des Wasserhaushaltes im Anaphylaktischen und im Histaminschock. Ztschr. f. exper. Med., 1933, **92**, 293.
31. Mann, F. C., The relation of the liver to metabolism. Harvey Lectures, New York, 1927, **23**, 49.
32. Frohlich, A., and Zak, E., Der Kreislauf in der Froschzunge unter pathologischen Bedingungen. Klin. Wchnschr., 1922, **1**, 1055.

33. Molitor, H., and Pick, E. P., Die Bedeutung der Leber für die Diurese. Arch. f. exp. Pathologie und Pharmakologie, 1923, 97, 317.
34. Soper, F. L., The pharmacopeia and the physician, treatment of yellow fever. J. A. M. A., 1942, 118, 374.
35. Krieger, V. I., and Kilvington, T. B., Antidiuretic substance in urine in relation to normal and toxaemic pregnancy. Med. J. Australia, 1940, 1, 575.
36. Shay, H., Kohm, R., and Fels, S. S., Some observations on changes in renal water output of the albino rat under the influence of a high fat diet. Gastroenterology, 1945, 4, 257.
37. Wintrobe, M. W., Relation of disease of the liver to anemia; type of anemia, response to treatment and relation of type of anemia to histopathologic changes in liver, spleen and bone marrow. Arch. Int. Med., 1936, 57, 289.
38. Best, C. H., and Taylor, N. B., The Physiological Basis of Medical Practice. 4th Ed., The William Wilkins & Co., Baltimore, Md., 1945.
39. Barcroft, J., Nisimaru, Y., and Ray, G. B., Observations on the time taken for corpuscles to traverse the liver. J. Physiol., 1932, 74, 44.
40. Roholm, K., and Iversen, P., Histopathology of acute infective hepatitis, changes in the liver in acute epidemic hepatitis (catarrhal jaundice) based on 38 aspiration biopsies. Acta Path. et Microbiol. Scandinav., 1939, 16, 427.
41. Lucké, B., and Mallory, T., The fulminant form of epidemic hepatitis. Am. J. Path., 1946, 22, 867.
42. Perera, G. A., The plasma volume in Läennec's cirrhosis of the liver. Ann. Int. Med., 1946, 24, 643.
43. Labby, D. H., The relation of plasma volume to the serum proteins in diseases of the liver. In preparation.
44. Hill, J. N., and Hausman, W., Liver deficiency anaemia in a case of acute infective hepatitis. Brit. M. J., 1943, 2, 262.
45. Labby, D. H., Kunkel, H. G., Shank, R. E., and Hoagland, C. L., Metabolic and therapeutic experiences in acute hepatic insufficiency: A study of 386 cases of acute infectious hepatitis. In preparation.
46. Lichtman, S. S., and Sohval, A. R., Clinical disorders with associated hepatic and renal manifestations, with especial reference to the so-called "hepato-renal syndrome." Am. J. Digest. Dis. and Nutrition, 1937, 4, 26.
47. Boyce, F. F., The Role of the Liver in Surgery. Charles C. Thomas, Baltimore, Maryland, 1941.
48. Gerritzen, F., Liver diuresis. Acta med. Scandinav., 1936, 89, 101.
49. Pick, E. P., The regulation of water metabolism. Harvey Lectures, New York, 1929, 25, 25.
50. Aldersberg, D., Zur Rolle der Leber im Wasserhaushalt. Wien. Arch. f. inn. Med., 1943, 25, 269.
51. Pick, E. P., Topics in the study of water metabolism. J. of Mt. Sinai Hosp., 1946, 13, 167.
52. Gilder, H., and Hoagland, C. L., Urinary excretion of estrogens and 17-ketosteroids in young, adult males with infectious hepatitis. Proc. Soc. Exper. Biol. and Med., 1946, 61, 62.
53. Robinson. F. H., and Farr, L. E., The relation between clinical edema and the excretion of an antidiuretic substance in the urine. Ann. Int. Med., 1940, 14, 42.
54. Ralli, E. P., Robson, J. S., Clarke, D. H., and Hoagland, C. L., Factors influencing ascites in patients with cirrhosis of the liver. J. Clin. Invest., 1945, 24, 316.
55. Labby, D. H., Shank, R. E., Kunkel, H. G., and Hoagland, C. L., The treatment of cirrhosis of the liver with intravenous crude liver extract. J. A. M. A. (In press.)
56. Fremont-Smith, F., The mechanism of edema formation. New England J. Med., 1932, 206, 1286.
57. Mellors, R. C., Muntwyler, E., Mautz, F. R., and Abbott, W. E., Changes of the plasma volume and "available (thiocyanate) fluid" in experimental dehydration. J. Biol. Chem., 1942, 144, 785.
58. Gilman. A., and Goodman, L., The secretory response of the posterior pituitary to the need for water conservation. J. Physiol., 1937, 90, 113.
59. Ingram, W. R., Ladd, L., and Benbow, J. T., The excretion of an antidiuretic substance and its relation to the hypo-thalamicohypophyseal system in cats. Am. J. Physiol., 1939, 127, 544.
60. Heller, H., and Urban, F. F., The fare of antidiuretic principle of post pituitary extracts *in vivo* and *in vitro*. J. Physiol., 1935, 85, 502.

THE DISTRIBUTION OF ASCORBIC ACID IN THE BLOOD

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In this paper we are reporting the results of a study of the distribution of ascorbic acid in the blood in human subjects and in guinea pigs.

EXPERIMENTAL

Fifty healthy medical students (47 males and 3 females) on self-selected diets and 50 patients (41 males and 9 females) in a municipal hospital were studied. Oxalated blood samples were collected under fasting conditions about 15 hours post-prandial. The ascorbic acid content of the whole blood and the plasma was determined by the method of Roe and Kuether (1). The concentration of ascorbic acid in oxalated whole blood and plasma was also determined by the same method in guinea pigs upon an apparently adequate diet (2) made up to contain different levels of ascorbic acid.

RESULTS

The results of our studies with healthy human subjects are shown graphically in Figure 1. Since all bloods were collected approximately 15 hours after food intake, the values may be considered as representing equilibrium conditions. The distribution ratio of ascorbic acid in the plasma to that in the whole blood follows a characteristic pattern. At whole blood levels below 0.6 mgm. per 100 ml., the plasma concentration was lower than the whole blood concentration in all cases. At whole blood levels ranging from 0.6 to 0.9 mgm. per 100 ml., the plasma concentration was either equal to, or slightly greater or less than, the whole blood concentration; in other words, at these levels, the plasma and whole blood concentrations approached each other very closely. At whole blood levels above 0.9 mgm. per 100 ml., the plasma concentration was higher than the whole blood concentration in all instances, except one.

The results shown in Figure 2 with 50 hospitalized patients suffering serious illness are in striking contrast to those obtained with healthy human subjects. In these patients, the ascorbic acid of the plasma was lower than that in the whole blood in all cases. The clinical diagnoses made in this group of patients were as follows: Pulmonary tuberculosis, hyperthyroidism, syphilis, glomerulonephritis, portal cirrhosis, hepatitis, diabetes mellitus, carcinoma, pernicious anemia with cellulitis, cystitis, pyelonephritis, duodenal ulcer, Addison's disease, rheumatoid arthritis, and cardiovascular heart disease.

In Figure 3 is shown the relation of the level of ascorbic acid in the plasma to that in the whole blood in 52 guinea pigs fed an apparently adequate diet containing different levels of ascorbic acid. The same pattern of distribution ratio of ascorbic acid in the plasma to that in the whole blood was observed in the guinea pigs as in our healthy human subjects. The results are not so clear-cut, however. We think this was due to the fact that smaller samples of blood were collected from the guinea pigs than from the human subjects, especially the scorbutic pigs, which probably influenced the precision of the analytical data obtained.

DISCUSSION

Pijoan and Eddy (3) found the ascorbic acid content of the plasma higher than that in the whole blood in 12 normal subjects with high ascorbic acid blood levels. Heinemann reported that the concentration of ascorbic acid is consistently higher in whole blood than in plasma or serum (4, 5), in fasting subjects. Stephens and Hawley (6) observed that the concentration of ascorbic acid was higher in the plasma than in the whole blood in 12 of 19 normal subjects. With 10 normal subjects having whole blood concentrations above 0.8 mgm. per 100 ml., Butler and Cushman (7) found the concentration of ascorbic acid in the plasma higher than in the whole blood in 3 subjects, lower in 4 subjects, and the same as in the whole blood in 3 subjects. With 8 subjects having whole blood concentrations lower than 0.23 mgm. per 100 ml., Kyhos, Sevringhaus, and Hagedorn (8) found the plasma level lower than the whole blood level in all cases; and, with 9 subjects having whole blood levels above 1.17 mgm. per 100 ml., there

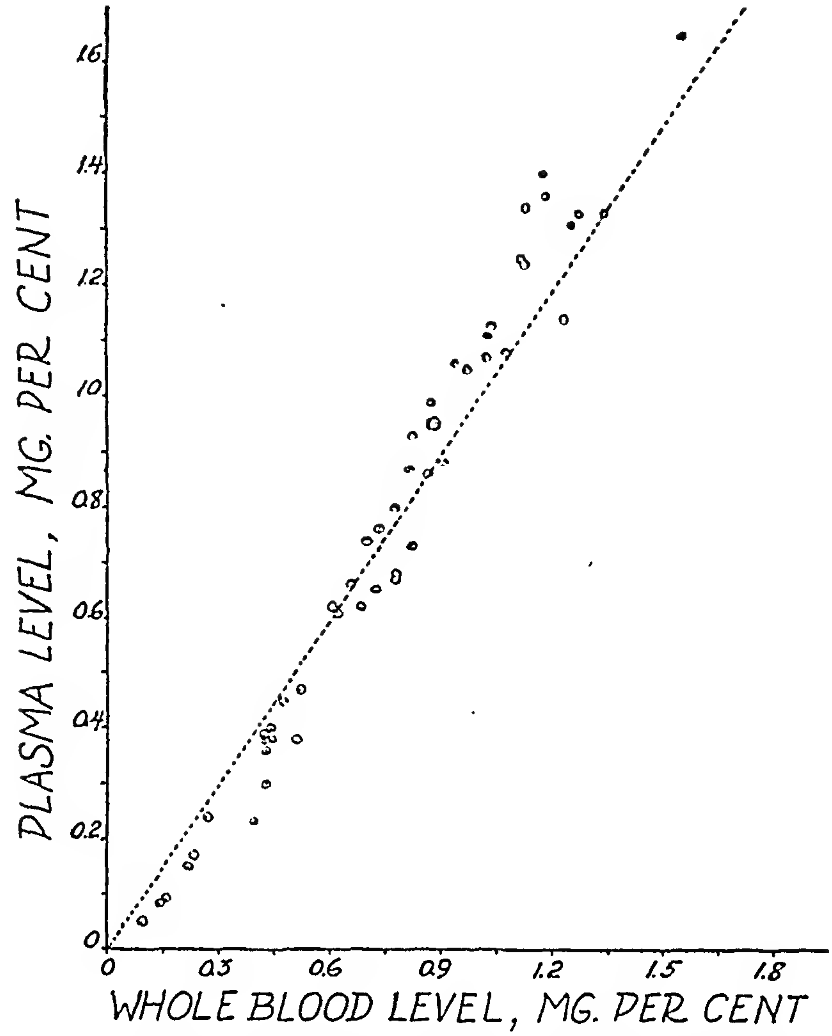

FIG. 1. THE RELATION OF THE LEVEL OF ASCORBIC ACID IN THE WHOLE BLOOD TO THAT IN THE PLASMA IN 50 HEALTHY HUMAN SUBJECTS

authors found the plasma content higher than the whole blood content in 2 instances and lower in 7 instances. Lubschez (9) made a study of whole blood and plasma concentrations of ascorbic acid in 63 subjects. She found that the majority of whole blood values were higher than the plasma values and that the relation between plasma and whole blood concentrations did not show any trend at different levels.

Our data upon subjects having high blood levels of ascorbic acid are in agreement with the work of Pijoan and Eddy (3); and our results with subjects having low blood levels agree with those data in the report of Kyhos, Sevringhaus, and Hagedorn (8) dealing with low blood levels. We are unable to explain the disagreement of our data with Heinemann's results, unless it is due to the different methods of analysis used. It is suggested that some of the opposite results in the literature may be reconciled by a recognition of the effect of the concentration of ascorbic acid in the blood upon its distribution between plasma and whole blood. It also appears desirable for data to be classified on the basis of whether the subjects are healthy or are suffering from some underlying disease that may alter the metabolism of vitamin C.

In our normal subjects the data show that the distribution of ascorbic acid between the plasma and the whole blood is related to the level of ascorbic acid in the blood. At high blood levels the plasma concentration is higher than the whole blood concentration; at intermediate blood levels the plasma and whole blood concentrations are approximately the same; and at low blood levels the plasma concentration is considerably lower than the whole blood concentration.

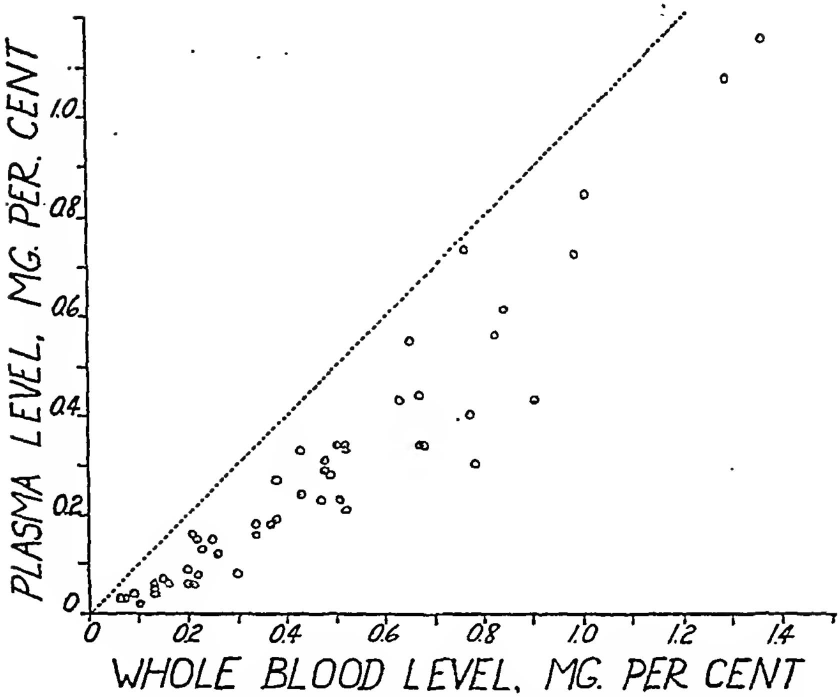

FIG. 2. THE RELATION OF THE LEVEL OF ASCORBIC ACID IN THE WHOLE BLOOD TO THAT IN THE PLASMA IN 50 PATIENTS

In guinea pigs upon the levels of ascorbic acid fed in our studies it has been observed that the concentration of ascorbic acid in the blood varies directly with the intake of this vitamin as shown in Figure 3 (2). In normal human subjects it is well-accepted that the blood level of ascorbic acid parallels the dietary intake of the vitamin up to a point where massive doses are administered (10 to 14). The unexplained effect of massive doses of ascorbic acid, observed by Wilson and Lubschez (14), was not present in our normal human subjects. In view of the evidence upon the relation of intake to the blood level of ascorbic acid and our observations upon the effect of blood level upon the distribution of ascorbic acid in blood, it is suggested that the change in the ratio of the concentration of ascorbic acid in the plasma to that in the whole blood in our normal subjects (Figures 1 and 3) was due to a parallel variation in the intake of ascorbic acid.

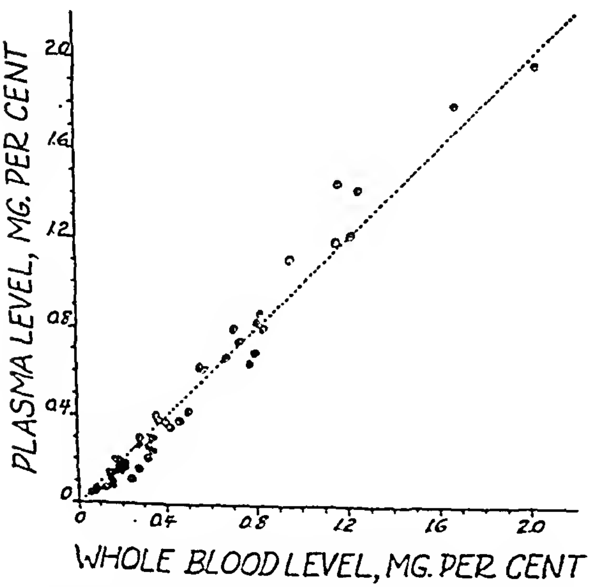

FIG. 3. THE RELATION OF THE LEVEL OF ASCORBIC ACID IN THE WHOLE BLOOD TO THAT IN THE PLASMA IN 52 GUINEA PIGS

The results of our studies with 50 patients, showing that the concentration of ascorbic acid in the plasma was lower than that in the whole blood in all instances, are of considerable interest. The concentration of the ascorbic acid in the plasma would be expected to be lower than that in the whole blood in patients with whole blood levels below 0.6 mgm. per 100 ml., since this distribution was observed in normal subjects with similar blood levels. However, the plasma con-

tent was lower than the whole blood content in 15 subjects with blood levels above 0.6 mgm. per 100 ml. Furthermore, comparison of Figure 2 with Figure 1 clearly shows that, at the same whole blood levels, the ratio of the plasma concentration to the whole blood concentration is considerably lower in the patients than in the healthy subjects. Therefore, in the patients, other factors, not in operation in the healthy subjects, contributed to the production of a concentration of ascorbic acid relatively lower in the plasma than in the whole blood. The most probable explanation of this finding is that in patients with serious illnesses there is a greater need for ascorbic acid because of the underlying pathologic processes, and this need is supplied by a more rapid withdrawal of the vitamin from the plasma than occurs in healthy subjects.

SUMMARY AND CONCLUSIONS

1. A study of the distribution of ascorbic acid in the blood in human subjects and in guinea pigs has been made.

2. In 50 fasting healthy human subjects, the following was observed: At whole blood levels of ascorbic acid below 0.6 mgm. per 100 ml., the plasma content was lower than the whole blood content; at whole blood levels about 0.6 to 0.9 mgm. per 100 ml., the concentration in the plasma was approximately the same as in the whole blood; at whole blood levels above 0.9 mgm. per 100 ml., the plasma content was higher than the whole blood content.

3. In 52 non-fasted guinea pigs the distribution of ascorbic acid between the plasma and the whole blood followed the same pattern as observed in healthy human subjects.

4. In 50 hospitalized fasting patients with various diseases, the concentration of ascorbic acid in the plasma was lower than that in the whole blood in all instances.

5. It is suggested that in normal subjects the change in the ratio of the ascorbic acid in the plasma to that in the whole blood is due to a parallel variation in the intake of ascorbic acid and that in patients both dietary intake and underlying pathologic processes operate to produce the resulting pattern of distribution of ascorbic acid in the blood.

We wish to express our thanks to Dr. S. Loube, of the Staff of the Gallinger Municipal Hospital, for selecting the clinical cases studied, and to R. W. Prichard, W. R. Strong, O. H. Christofferson, and N. Goldstein, of the George Washington University Medical School, for collecting the blood samples and laboratory data of the patients.

BIBLIOGRAPHY

1. Roe, J. H., and Kuether, C. A., The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., 1943, 147, 399.
2. Kuether, C. A., Telford, I. R., and Roe, J. H., The relation of the blood level of ascorbic acid to the tissue concentrations of this vitamin and to the histology of the incisor teeth in the guinea pig. J. Nutrition, 1944, 28, 347.
3. Pijoan, M., and Eddy, E., Ascorbic acid content of red cells and plasma. J. Lab. & Clin. Med., 1937, 22, 1227.
4. Heinemann, M., On the partition of ascorbic acid between plasma and erythrocytes. Acta Brev. Neerland, 1936, 6, 139.
5. Heinemann, M., The distribution of ascorbic acid between cells and serum in relation to its urinary excretion. J. Clin. Invest., 1938, 17, 751.
6. Stephens, D. J., and Hawley, E. E., The partition of reduced ascorbic acid in blood. J. Biol. Chem., 1936, 115, 653.
7. Butler, A. M., and Cushman, M., Distribution of ascorbic acid in the blood and its nutritional significance. J. Clin. Invest., 1940, 19, 459.
8. Kyhos, E. D., Sevringhaus, E. L., and Hagedorn, D. R., Large doses of ascorbic acid in treatment of vitamin C deficiencies. Arch. Int. Med., 1945, 75, 407.
9. Lubschez, R., Studies in ascorbic acid with special reference to the white layer. I. Description of method and comparison of ascorbic acid levels in whole blood, plasma, red cells, and white layer. J. Clin. Invest., 1945, 24, 573.
10. Farmer, C. J., and Abt, A. F., Ascorbic acid content of blood. Proc. Soc. Exper. Biol. & Med., 1935, 32, 1625.
11. Greenberg, L. D., Rinehart, J. F., and Phatak, N. M., Studies on reduced ascorbic acid content of the blood plasma. Proc. Soc. Exper. Biol. & Med., 1936, 35, 135.
12. Thysell, T., Diet, blood ascorbic acid, and the capillary bleeding test. Acta Paediat., 1939, 26, 481.
13. Prunty, F. T. G., and Vass, C. C. N., The assessment of vitamin C nutrition in man. Biochem. J., 1943, 37, 623.
14. Wilson, M. G., and Lubschez, R., Studies in ascorbic acid with special reference to the white layer. II. The relation of intake to blood levels in normal children and the effect of acute and chronic illness. J. Clin. Invest., 1946, 25, 428.

FACTORS CONCERNED IN THE CIRCULATORY FAILURE OF ADRENAL INSUFFICIENCY

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An increase in the packed cell volume of the blood is a typical finding in the crisis of adrenal insufficiency. Swingle, Vars and Parkins (1), by direct and indirect (dye method) measurements of blood and plasma volumes, demonstrated that a reduced plasma volume was the main cause of the increase in the relative volume of red cells. In a series of papers, Swingle and his coworkers (2, 3, 4, 5) postulated the importance of impairment of capillary tone and consequent increase in capillary permeability to protein as an independent factor in the causation of reduced plasma volume and of circulatory failure in crisis.

On the other hand, Loeb (6, 7) and Harrop (8, 9) focused their attention on the changes in the elimination of sodium and potassium by the kidneys and on the changes in electrolyte content of the blood and their logical consequences upon the body fluids. A net loss of sodium could cause a depletion of the plasma volume either by carrying away an equivalent volume of extracellular fluid or simultaneously causing a shift of water from the extracellular to the intracellular compartment because of the reduction in effective osmotic pressure of the extracellular fluid. A net retention of potassium might be expected to have a similar net effect, namely, a shift of fluid from the extracellular to the intracellular compartments. According to Harrison and Darrow (10), "Shifts in body fluids do not play a deciding role in the genesis of symptoms following adrenalectomy in the rat." Others, however, have found that slight hydration of muscle does occur in the rat (10) and in the dog (11, 12, 13) in adrenal insufficiency, being more pronounced in the latter animal.

If the plasma volume were diminished as a result of any or all of these aberrations in the regulation of electrolytes, it should be accompanied by a decrease in the interstitial fluid (extracellular tissue fluid). And, indeed, since interstitial fluid is considered to be a labile reservoir for the plasma volume, one might even expect a greater relative change in the interstitial volume than in the plasma volume. On the other hand, if decreased capillary tone and increased capillary permeability to protein were the only important factors in the reduction of plasma volume, the volume of interstitial fluid should show a concomitant increase. If both electrolytes and capillary factors were important and independent consequences of adrenal insufficiency, the changes in interstitial fluid might well be very variable. The interstitial fluid volume might sometimes increase and sometimes decrease. If the permeability of capillaries to protein increased as a result of changes in electrolytes, *e.g.* a change in ratio of sodium to potassium, a decrease in the interstitial fluid volume should be the rule, but it might be very variable in extent. A study of interstitial fluid volumes in adrenal insufficiency produced under different circumstances might thus throw some light on the relative importance of the various factors which have been postulated. So far, the only study of the interstitial fluid of the body as a whole in adrenal insufficiency is that of Harrop (9) on one adrenalectomized dog, which showed a striking decrease, supporting the importance of the electrolyte changes in the production of crisis.

In the present study, we have measured the plasma volume by the blue dye T 1824 and the interstitial fluid by sulfocyanate in adrenalectomized dogs during adequate maintenance with hormone and during crisis after deprivation of hormone. We have also watched for evidence of still another factor in the circulatory failure of crisis, namely, impairment of the function of the heart. Cleghorn and his coworkers have stressed the importance of cardiac failure in adrenal insufficiency in cats, in dogs, and in patients with Addison's disease (14, 15). It may occur in the absence of hemoconcentration or changes in the electrolyte pattern of the blood and quite apart from

over-treatment with desoxycorticosterone, as reported by Ferrebee *et al.* (16). Loeb (17), too, has reported cardiac failure in patients apparently not over-treated and McGavack (18) has drawn attention to the small size of the heart in Addision's disease.

A preliminary account of these experiments has been reported previously in abstract form (19).

METHODS

The following series of experiments were conducted:

Series 1. Seven adrenalectomized dogs were studied while adequately maintained with cortin (1 ml. twice daily) on a diet containing approximately 2.0 per cent NaCl. The studies were repeated on the same dogs during crisis after deprivation of cortin.

Series 2. Three of these dogs, after recovery from the experiments of Series 1, were maintained on a low salt diet (about 1.0 per cent NaCl) and larger amounts of cortical extract (4 ml. twice daily) and the studies repeated and again repeated during crisis after deprivation of hormone.

Series 3. Two dogs were maintained on cortin (2 ml. twice daily) and desoxycorticosterone acetate (1.25 mgm. once daily) with low salt diet and the same studies repeated before and during crisis after deprivation of both substances.

Plasma volumes and available fluid volumes were measured by the method of Gregersen and Stewart (20), modified for use with the Evelyn photoelectric colorimeter. Available fluid volume is defined as the volume in which sulfocyanate would be dissolved in the same concentration as in the plasma. Available fluid $(A) = Q/S$, where $Q =$ quantity of sulfocyanate injected and S its concentration in serum. This volume includes plasma volume and approximately half the red cell volume, in addition to some volume corresponding roughly to the volume of extracellular tissue fluids. The latter hypothetical entity we have called the Interstitial Volume (I.V.). It is calculated by the formula I.V. $= 0.95(A-P-.5C)$, where $P =$ plasma volume and $C =$ erythrocyte volume. No particular significance is attached in this paper to the absolute value of the Interstitial Volume as defined above. In spite of some disagreement (21, 22) as to the meaning of the apparent volume of distribution of sulfocyanate in the body, it is generally conceded that changes in its magnitude represent changes in a fluid compartment of the body in which many electrolytes diffuse rather freely. Determinations were done during the period of maintenance and after the development of severe adrenal insufficiency when loss of appetite, vomiting, weakness, or bradycardia had developed. All animals were restored by energetic therapy after the determinations in insufficiency and were used again. Blood was drawn from the jugular vein in an oiled syringe and delivered under oil. Urine was analyzed for chloride in daily specimens, for sodium in 2-day samples. The analytical methods were: sodium [Butler and Tuthill (23)], potassium [Shohl and Bennett (24)], chloride [modified Volhard Harvey (25)], non-protein nitrogen [Folin and Dennis (26)]. Packed cell volume determinations were made on heparinized blood spun to a constant volume at 3,000 r.p.m. No calculation was made for plasma trapped between the cells.

RESULTS

The observations on all 3 series of experiments are shown in Table I. Table II gives the averages of the principal findings in each series. The average plasma volume in Series I during maintenance agrees well with the average value for normal dogs (48 ml. per kgm.) (27).

In Series II the average plasma volume during

TABLE I

Dog no.	Body wt.	Packed cell volume	Cardiac rate	Plasma volume	Interstitial volume	Serum Na	Serum K
	kgm.	*per cent*		*ml.*	*ml.*	*meq. per l.*	*meq. per l.*
Series I (cortin + extra NaCl)							
1. A	16.60	33.0	110	715	3,910	143	5.20
B	16.30	42.0	52	550	3,820	130	8.90
2. A	8.50	30.0	100	500	2,310	142	5.90
B	8.15	39.0	40	381	2,230	133	8.00
3. A	10.75	32.0	108	459	2,400	140	6.40
B	10.24	39.0	110	325	1,910	132	7.40
4. A	11.20	45.0	110	610	2,630	137	4.65
B	10.60	49.0	52	394	2,410	128	8.90
5. A	16.56	48.0	120	623	2,880	141	5.40
B	15.45	55.0	66	393	2,780	127	7.90
7. A	11.50	46.0	110	550	2,288	140	5.60
B	11.04	57.6	46	377	2,150	125	
8. A	10.90	38.0	112	585	2,781	151	5.40
B	9.70	52.0	122	373	2,252	122	7.10
Series II (cortin only)							
1. A	17.10	35.5	116	610	3,630	142	5.60
B	17.00	42.0	125	537	3,120		8.20
2. A	8.60	36.0	112	452	1,787	144	5.40
B	8.33	45.0	40	326	1,710		9.40
4. A	11.60	39.0	130	580	2,460	138	5.60
B	11.46	43.0	48	491	2,380		8.40
Series III (cortin plus desoxycorticosterone)							
2. A	8.90	29.0	85	635	2,440	156	
B	8.40	51.0	44	373	1,431	139	
6. A	10.10	33.0		552	2,342	146	
B	9.60	44.0	55	300	2,290	123	

A—Control. B—Crisis.

Zn Metobolism Studied by Means of the Radioactive Isotope $_{30}Zn^{65}$. BERT L. VALLEE and REX G. FLUHARITY, Boston, Mass. (Introduced by John G. Gibson, 2nd).

External scanning with a gamma ray Geiger-Müller counter has shown that, following i.v. administration, $_{30}Zn^{65}$ becomes widely distributed throughout the entire body of the normal dog. The normal range of zinc content of organs has been determined in normal dogs by an improved dithizone method. The ranges, in completely perfused organs, in gamma per gram (wet weight), are: heart and great vessels 15–20; respiratory organs 10–20; digestive organs 10–60; genito-urinary tract 20–50; muscle 40–50; cortical bone 40–50; C.N.S. (brain) 10–15; endocrines 20–100; lymph glands 15–100; liver 50–60; spleen 10–20; marrow 60–80; plasma 2–4 (per cc.); whole blood 7–9. Simultaneous measurements of Zn^{65} and total zinc content have not shown selective localization of the element in any of the above organs.

The complete separation of white cells from blood can be effected by floatation of heparinized whole blood on an isosmotic solution of bovine serum albumin of specific gravity of approximately 1.079. The normal zinc content of white cells is 1×10^{-2} gamma and of red cells 1×10^{-4} gamma per million cells, respectively. Following the intravenous injection of Zn^{65} the isotope becomes concentrated within the circulating white cells in amounts sufficient for accurate x-ray detection in the leucocyte mass separated from 15 cc. of whole blood (1.0 to 2.0×10^{9} w.b.c.).

Blood, white cells and tissue samples are dry ashed, and the zinc extracted with dithizone for colorimetry. The zinc is removed from the dithizonate with sulfuric acid and electroplated from an alkaline solution with added carrier zinc on a copper disk. The x-ray counting procedures are similar to those used to measure Fe^{55}.

These procedures have opened the way to a study of overall zinc metabolism in addition to the physiology of leucocytes.

Late Manifestations of Epidemic Infectious Hepatitis. WADE VOLWILER and JOSEPH A. ELLIOT Boston, Mass. (Introduced by Chester M. Jones).

Accurate and complete observations on the late manifestations of epidemic infectious hepatitis are relatively rare. Needle liver biopsy and multiple laboratory tests were carried out on twelve army and civilian patients having symptoms of this disease four months to twenty-one years after the initial illness. A small group with continued mild jaundice showed normal liver histology and normal liver function tests with no evidence of a hemolytic process. Of the remaining eight, all showed definite histologic evidence of a persisting inflammatory process associated with clinical symptoms of varying severity. Abnormal physical signs and symptoms did not correlate well with the degree of observed histologic damage. Bromsulfalein excretion was the only consistently abnormal hepatic function test in this group. The sedimentation rate was usually elevated, in contrast to the normal values commonly seen in the acute phase of the disease. Flocculation tests and quantitative 24-hour urine urobilinogen determinations frequently did not reflect the histologic abnormalities. Two patients with severe laboratory hepatic functional impairment were found to have developed fibrosis in association with still active inflammation. Colored photomicrographs of biopsy specimens will be shown.

The Hemodynamics of Rapid Changes in Cardiac Output in Man. JAMES V. WARREN, New Haven, Conn., and (by invitation) EMMETT S. BRANNON and FREDERICK W. COOPER, JR., Atlanta, Ga.

Patients with large arteriovenous fistulas offer an unusual opportunity to study the hemodynamic alterations associated with rapid changes in cardiac output. Ballistocardiographic studies on fifty such patients during the recent war indicated that the resting cardiac output was often abnormally high, and on occlusion of the fistula fell to a normal value. Further observations on the nature of these circulatory alterations have been made in 14 patients utilizing the technique of right heart catheterization.

The present observations confirmed the ballistocardiographic findings. In the patients with a large stroke volume, the arteriovenous oxygen difference (determined from right heart blood) was found to be low, with blood draining the area of the fistula giving evidence of an even lower arteriovenous oxygen difference.

Although the venous pressure near the fistula and distal to it was elevated in some instances, the right atrial pressure was normal in all patients where it was studied. Although compression of the fistula produced a striking fall in cardiac output in some patients, there was no change in atrial pressure. Experimental lowering of the atrial pressure failed to produce significant change in cardiac output, thus indicating that minor undetectable changes in atrial pressure were not responsible for the changes in cardiac output.

The right ventricular and pulmonary arterial pressure were recorded in four patients during opening and closing of the fistula. Despite considerable change in cardiac output, only minimal change in pressure and pulse contour occurred.

These observations give further support to the belief that wide variations in cardiac output may occur in the absence of significant change in atrial pressure. The constancy of pulmonary arterial pressure indicates a rapid, presumably reflex, alteration in pulmonary vascular resistance associated with changes in cardiac output.

Kidney Function Studies in Adrenal Insufficiency. CHRISTINE WATERHOUSE and E. HENRY KEUTMANN, Rochester, N. Y. (introduced by William S. McCann).

The glomerular filtration rate (mannitol), renal plasma flow (PAH), and maximum tubular excretory capacity (PAH) were studied in six male and six female patients with adrenal insufficiency. Nine had Addison's Disease and three had pan-hypo-pituitarism. All were in good clinical condition at the time of study.

All female patients had markedly reduced maximum tubular excretory capacity. This function was normal in all males except two patients who had hypertension. These results suggest that maintenance of approximately

normal amounts of functioning tubular tissue occurs only in males.

The reduced tubular function in females was not corrected by desoxycorticosterone acetate, testosterone propionate, thyroid, aqueous adrenal cortex extract in large doses or pork adrenal cortex extract in oil.

The renal plasma flow was normal in two men but in all other patients it was below normal. Neither clinical nor laboratory data clarified this difference entirely but the patients with Addison's Disease receiving desoxycorticosterone acetate had a greater decrease of renal plasma flow than reduction in the ability of the tubules to excrete PAH (low ratio R.P.F/Tm). This suggests that the post glomerular arterioles are constricted by the hormone.

All patients had reduced glomerular filtration rates at all times. While this defect may be exaggerated in crisis and improvement is caused with restoration of electrolyte deficit by administration of salt, adrenal cortex extract or desoxycorticosterone, normal values are not attained.

Correlation between the Effect of Quinidine Sulfate on the Rate of the Circus Movement in Auricular Fibrillation and its Concentration in the Blood Plasma. René Wégria, New York, N. Y. (Introduced by Alexander B. Gutman).

Patients with auricular fibrillation were given orally single and repeated doses of quinidine sulfate. The concentration of the drug in the plasma and the intensity of its effect on the rate of the circus were followed.

In 7 studies in which one single dose (0.4 gm. to 0.8 gm.) of quinidine was given, the intensity of the cardiac effect of the drug and its plasma concentration were found to be grossly parallel but not parallel in a strictly quantitative manner. Indeed, discrepancies between intensity of cardiac effect and plasma concentration were found, the plasma level of the drug decreasing faster than the intensity of its cardiac effect. In 3 studies in which repeated doses (0.4 gm. every 2 hours for 3 or 4 doses) were given, there was a less marked discrepancy between plasma concentration and intensity of cardiac effect. Several possible explanations which may account for the discrepancies between the plasma level of the drug and the intensity of its cardiac effect, were investigated.

Observations on a Perfusion Method for Reducing Azotaemia in Temporary Renal Failure. Donald M. Whitlaw, Toronto, Canada (Introduced by W. Hurst Brown).

Perfusion of the peritoneum was carried out for six days in a patient with extreme oliguria of five days duration due to sulfathiazole nephrosis. Perfusion fluid was solution "A" of Abbott and Shea with added heparin, penicillin and streptomycin. Perfusion rate was 1.5 liters per hour for two days and 0.4 liters an hour for the four subsequent days. Nonprotein nitrogen was reduced from 145 mg per cent to 82 mg per cent in the first two days and maintained at that level. Carbon dioxide combining power was maintained between 18 and 21 mEq. per liter. Edema was at first reduced but later reappeared due to loss of protein in the perfusate. A total of 71.35 liters of perfusate was recovered which contained 46.4 gm. of nonprotein nitrogen and 178 gm. of protein. Concentration of nonprotein nitrogen in the perfusate averaged 70 mg. per cent and varied directly with the length of time that the fluid remained in the peritoneal cavity. Diuresis began on the second day of perfusion and reached 5.5 liters on the last day. Twenty-five days later urine output was normal. Urinary specific gravity remained fixed between 1.010 and 1.012. Urea clearance was 25 cc., nonprotein nitrogen was 42 mg per cent. No peritoneal infection occurred.

The Effects of Splanchnicectomy upon Hepatic Function and Blood Flow in Hypertensive Patients. Robert W. Wilkins, (by invitation) James W. Culbertson and Franz J. Ingelfinger, Boston, Mass.

Hepatic function and blood flow were estimated in hypertensive patients before, and again early after sympathectomy (Smithwick) by the method of Bradley *et al.* Briefly this involves the measurement of BSP concentration in serial pairs of samples of peripheral and hepatic (catheter method) venous blood, during a constant intravenous infusion of the dye. In the presence of a constant, or constantly changing, peripheral blood level of BSP calculations of total removal rate may be made. The hepatic extraction of BSP is represented by the peripheral-hepatic venous difference. Hepatic blood flow is estimated by dividing the total removal rate by the extraction.

Preoperative values for total BSP removal rate and estimated hepatic blood flow fell within the normal range. Postoperative measurements revealed a marked decrease in total removal rate of BSP, usually associated with a marked decrease in hepatic extraction. The data indicated that hepatic blood flow was markedly increased when resting blood pressure was unchanged after splanchnicectomy, whereas it was increased only slightly or not at all when blood pressure was lowered. These results suggest that sympathectomy markedly decreases the resistance to blood flow through sizable portions of the splanchnic beds draining through the liver.

The cause of the decrease in total BSP removal rate after sympathectomy is not clear, but probably is not a non-specific postoperative debility, since no decrease occurred after other major surgical procedures. Perhaps it is related to the increase in hepatic blood flow.

How Should Thyrotoxicosis Be Treated? Robert H. Williams and (by invitation) Samuel P. Asper, Jr., Walter F. Rogers, Jr., William H. Daughaday and Beverly T. Towery, Boston, Mass.

The increased availability of many effective and relatively safe antithyroid drugs and of radioactive iodine has made it necessary, in choosing the best treatment in thyrotoxicosis, to give individual consideration to the problems of each patient. As a result of treating 320 thyrotoxic patients with from 1 to 16 thiourea or aminobenzene derivatives, of following 28 individuals treated with radioactive iodine, of observing 155 patients subjected to thyroidectomy, and on the basis of the experiences reported by others, it has been possible to draw

tentative conclusions regarding the choice of therapy in this disease.

The antithyroid drugs are desirable in a large proportion of individuals because of the simplicity of administration, availability, inexpensiveness, regulability, safety, relative lack of physical or mental discomfort, and lack of damage to the thyroid gland and surrounding structures. The compounds tested clinically in this clinic are: tetramethylthiourea, diethylthiourea, thiourea, 2-thiobenzimiazoldine, aminothiazol, thiothymine, thiobarbital, thiouracil and 6-methyl-, 6-propyl-, 6-cyclopropyl-, 6-butyl-, 6-isobutyl-, thiouracil. p-Aminobenzoic acid and its 3,5-diiodo derivative, as well as 3,5-diiodo-o-aminobenzoic acid have also been used. Of these compounds, 6-propyl-, 6-cyclopropyl-, and 6-butyl- thiouracil are the ones of choice. They have a high degree of effectiveness and the complications from their use are very infrequent. The pharmacology of the derivatives of thiouracil was found to be similar to that of thiouracil, but they tend to be broken down more slowly and are concentrated more in the thyroid gland, in rats. Substituents in the 6-position of thiouracil bearing an odd number of carbon atoms (methyl, propyl and amyl derivatives) persist in the carcass in greater concentration and in the thyroid gland in less concentration than was found with ones containing an even number of carbon atoms (ethyl and butyl). The antithyroid activity of thiouracil and propylthiouracil is enhanced by the simultaneous administration of bromide, fluoride and chloride, presumably due to competitive reactions of these halides with iodine.

Radioactive iodine is quite useful in thyrotoxic patients who have large goiters, individuals who cannot or will not take prolonged therapy, and ones who have not responded satisfactorily to antithyroid drugs or to thyroidectomy. It is also useful in certain patients with carcinoma of the thyroid gland which has metastasized.

It is not necessary for a large proportion of patients to be subjected to thyroidectomy, but this procedure is desirable in certain individuals with a large thyroid gland, in ones who may likely have carcinoma of the thyroid and in the small number of subjects who cannot be satisfactorily treated otherwise.

Sustained Contraction of the Diaphragm, the Mechanism of a Common Type of Dyspnea and Precordial Pain. STEWART WOLF, New York, N. Y.

Complaints of respiratory distress characterized by inability to get a full breath were found to occur commonly among anxious individuals and among those who did not obviously display anxiety. By discussion of situational conflicts, attacks were induced in 17 subjects during flouroscopic observation. In each instance a characteristic disorder of diaphragmatic function was observed. Inspiration became jerky and the excursion of the diaphragm exceeded that in expiration. The diaphragm thus assumed a progressively lower position. When its contractile state was such that an adequate inspiratory excursion was no longer possible, dyspnea occurred with a feeling of inability to take a breath. Frequently pain in the precordium, elsewhere in the chest, and in the shoulder accompanied such sustained diaphragmatic contraction.

Three subjects had attacks accompanied by pallor, sweating, fall in blood pressure and angor animi, simulating superficially the syndrome of coronary occlusion.

In seven of the subjects occlusion of the cardiac end of the esophagus during diaphragmatic spasm was demonstrated with barium. Occasionally esophageal occlusion occurred when the increase in contractile state of the diaphragm was insufficient to impair ventilation of the lungs and cause dyspnea. Such patients also complained of difficulty in swallowing.

TABLE II

Averages

	Plasma volumes		Interstitial volumes		Decrease in absolute volumes	
	Control	Crisis	Control	Crisis	P.V.	I.V.
	ml. per kgm.	*ml. per kgm.*	*ml. per kgm.*	*ml. per kgm.*	*per cent*	
Series I	48.3	35.3	227	218	30.9	9.0
Series II	46.1	37.9	211	202	22.8	7.2
Series III	63.0	37.8	253	200	43.5	21.8

maintenance was slightly lower, while in Series III it was distinctly higher than the average for normal dogs. In crisis the plasma volume was invariably reduced. Expressed as ml. per kgm. of body weight in crisis, the average plasma volume in all 3 series is strikingly similar.

Interstitial fluid volumes (I.V.) in Series I and II were within normal limits during maintenance but higher than average for the 2 dogs receiving desoxycorticosterone (Series III). In all cases the interstitial volume was reduced in crisis but much less in relation to its original value than was the plasma volume. In some cases there was no reduction in the I.V. relative to body weight, although a small absolute decrease had occurred.

In all but 3 experiments, bradycardia developed during crisis.

In the dogs on the high salt diet, crisis occurred 11 to 13 days after stopping cortin. The dogs on low salt diets went into crisis on the third to the fifth day after stopping cortin.

Sodium and potassium levels in the serum showed the usual alterations. The total urinary excretion of electrolytes and water was followed in several animals, but control of total intake and output was hardly rigorous enough to allow us to draw definite conclusions as to the relative importance of electrolyte and water loss versus fluid redistribution within the body as contributory factors to the reduction of extracellular fluid. In the animals maintained without added salt, the increased excretion of sodium chloride and water was sufficiently great to account for most of the reduction in extracellular fluid.

In the dogs on the diet with extra sodium chloride a net loss of sodium and chloride was not obvious from the primary findings but might have occurred. On these animals, however, one striking observation was made which appears to have escaped notice heretofore. After deprivation of cortin, the intake of water and output of urine increased markedly, as if in the absence of cortin there was some hindrance to the excretion of urine with a high concentration of salt.

TABLE III

Water intake and urinary volumes and electrolytes on constant diet

A. 2.0 per cent NaCl diet—Average daily amounts for 7 days before, and 7 days after withdrawal of cortin.

Dog. no.	Water intake	Urine output	Sodium output	Chloride output
	ml.	*ml.*	*meq.*	*meq.*
1. before	718	420	62	62
after	823	513	76	70
2. before	603	243	40	42
after	757	441	47	45
3. before	525	241	53	54
after	553	299	56	52
4. before	738	348	73	73
after	969	475	74	72

B. 1.0 per cent NaCl diet—Average daily amounts for 3 days before and after withdrawal of cortin.

Dog. no.	Water intake	Urine output	Sodium output	Chloride output
2. before	384	147	7	8
after	281	237	35	28
6. before	820	450	11	18
after	443	273	30	33

DISCUSSION

The observation that interstitial fluid volumes are reduced in crisis, together with the well known fact which has been confirmed in these experiments that a high salt diet delays the onset of crisis, may be taken as evidence of the importance of electrolyte disturbances as a contributory factor in the production of crisis. Since, however, changes in the interstitial volume are variable and often slight, our results must be regarded as giving some support to Swingle's hypothesis of increased capillary permeability to protein.

In another communication we plan to present data which show that there is a considerable loss of protein, especially albumin, from the circulating plasma during the development of adrenal insufficiency. This loss must have been slow, for the concentration-time curves of the blue dye used in the determinations of blood volume did not de-

cline more rapidly in crisis than in control experiments. Our experiments contribute no evidence on the question of whether the capillary changes may be due to changes in the ratio of sodium and potassium in the plasma or are a manifestation of some effect of cortin quite independent of actions on sodium and potassium, either as to their renal excretion or distribution between intracellular or extracellular fluids.

If the average decrease in plasma volume in crisis is only about 30 per cent and the decrease in total blood volume much less (17 per cent according to our calculations), then we are forced to the conclusion that the circulatory failure of crisis must be due to something more than diminished blood volume. The extra factor may well be cardiac damage, as evidenced by the frequent occurrence of bradycardia. Dogs with intact adrenals will stand bleeding which reduces their blood volume by 45 per cent (28, 30). More recently blood volume measurements have been made by the dye method on humans in shock from burns and hemorrhage (29, 31). The reductions in blood volume compatible with recovery seem to be considerably greater than we have found in dogs in crisis. Cardiac failure would be a factor which would enhance the gravity of a modest decline in blood volume.

Evidence regarding the shift of fluid from extracellular to intracellular spaces is provided in these experiments only by changes in body weight of the animals. The loss in body weight in the 12 experiments averaged 500 grams. The estimated loss of plasma and interstitial fluid averaged 440 grams. In the 12 hours prior to crisis the animals usually ate and drank nothing. During that time the net loss of weight in the form of exhaled H_2O and CO_2 (less O_2 retained) might have amounted to 200 grams. If the decrease in extracellular fluid all represented urinary (and fecal) loss, the total weight loss should have been 640 grams. It seems permissible to postulate an average shift of about 140 grams of extracellular fluid into the intracellular space.

CONCLUSIONS

Our data on the whole support the hypothesis that in the crisis of adrenal insufficiency, loss of interstitial fluid contributes significantly to a decrease in plasma volume but does not account for all of it. Loss of protein from the plasma may also be a factor in reducing the plasma volume.

The reduction in circulating plasma volume seems insufficient to account for the circulatory failure in crisis. Cardiac failure and possibly loss of capillary tonus may contribute to the circulatory failure.

The loss of interstitial fluid seems to be mostly by renal excretion but some shift to intracellular spaces may also occur.

SUMMARY

1. In 12 experiments on 8 dogs, plasma volumes measured by the dye method in the crisis of adrenal insufficiency were about 30 per cent below normal on the average, while the total blood volume calculated from the plasma volume was only about 17 per cent below normal.

2. Interstitial fluid volumes measured by the sulfocyanate method in crisis were always reduced, but relatively much less than the plasma volumes, *viz.* about 5 per cent on the average with much variation.

3. The decrease in plasma volume may be attributed to at least 2 factors: (*a*) loss of total extracellular fluid as a result of loss of sodium and retention of potassium; (*b*) reduction in plasma proteins and consequent loss of plasma to the extravascular spaces.

4. Cardiac failure as evidenced by a high incidence of bradycardia is a more important factor in crisis than is usually recognized. It may be responsible, together with decreased capillary tone, for the severe circulatory failure associated with the apparently moderate decrease in blood volume.

5. In those experiments in which crisis after deprivation of cortin was delayed by a high salt diet, there was evidence suggesting inability of the kidneys to produce urine of high salt content.

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BIBLIOGRAPHY

1. Swingle, W. W., Vars, H. M., and Parkins, W. M., A study of the blood volume of adrenalectomized dogs. Am. J. Physiol., 1934, 109, 488.
2. Swingle, W. W., Parkins, W. M., Taylor, A. R., and

Hays, H. W., The influence of adrenal cortical hormone upon electrolyte and fluid distribution in adrenalectomized dogs maintained on a sodium and chloride free diet. Am. J. Physiol., 1937, 119, 684.

3. Parkins, W. M., Taylor, A. R., and Swingle, W. W., A comparative study of sodium, chloride and blood pressure changes induced by adrenal insufficiency, trauma and intraperitoneal administration of glucose. Am. J. Physiol., 1935, 112, 581.
4. Swingle, W. W., Parkins, W. M., Taylor, A. R., and Hays, H. W., Relation of serum sodium and chloride levels to alterations of body water in the intact and adrenalectomized dog, and the influence of adrenal cortical hormone upon fluid distribution. Am. J. Physiol., 1936, 116, 438.
5. Swingle, W. W., Parkins, W. M., Taylor, A. R., and Hays, H. W., A study of the circulatory failure and shock following trauma to the healthy vigorous adrenalectomized dog. Am. J. Physiol., 1938, 124, 22.
6. Loeb, R. F., Atchley, D. W., Benedict, E. M., and Leland, J., Electrolyte balance studies in adrenalectomized dogs with particular reference to the excretion of sodium. J. Exper. Med., 1933, 57, 775.
7. Loeb, R. F., The adrenal cortex. J. A. M. A., 1935, 104, 2177.
8. Harrop, G. A., Soffer, L. J., Ellsworth, R., and Trescher, J. H., Studies on the suprarenal cortex. III. Plasma electrolytes and electrolyte excretion during suprarenal insufficiency in the dog. J. Exper. Med., 1933, 58,. 17.
9. Harrop, G. A., The influence of the adrenal cortex upon the distribution of body water. Bull. Johns Hopkins Hosp., 1936, 59, 11.
10. Harrison, H. E., and Darrow, D. C., The distribution of body water and electrolytes in adrenal insufficiency. J. Clin. Invest., 1938, 17, 77.
11. Darrow, D. C., Harrison, H. E., and Taffel, M., Tissue electrolytes in adrenal insufficiency. J. Biol. Chem., 1939, 130, 487.
12. Muntwyler, E., Mellors, R. C., Mautz, F. R., and Mangun, G. H., Electrolyte and water equilibria in the dog. II. Electrolyte and water exchange between skeletal muscle and blood in adrenal insufficiency. J. Biol. Chem., 1940, 134, 367.
13. Clarke, A. P. W., and Cleghorn, R. A., Chemical studies of tissue changes in adrenal insufficiency and traumatic shock. Endocrinology, 1942, 31, 597.
14. Hall, G. E., and Cleghorn, R. A., Cardiac lesion in adrenal insufficiency. Canad. M. A. J., 1937, 39, 126.
15. Cleghorn, R. A., Therapeutic Symposium: Treatment of Addison's disease. G. W. Thorn. Discussed by Wilder, R. M., Thompson, W. O. and Cleghorn, R. A. J. Clin. Endocrinol., 1941, 1, 85.
16. Ferrebee, J. W., Ragan, C., Atchley, D. W., and Loeb, R. F., Desoxycorticosterone esters. Certain effects in the treatment of Addison's disease. J. A. M. A., 1939, 113, 1725.
17. Loeb, R. F., Adrenal insufficiency. Bull. New York Acad. Med., 1940, 16, 347.
18. McGavack, T. H., Changes in heart volume in Addison's disease and their significance. Am. Heart J., 1941, 21, 1.
19. Clarke, A. P. W., Cleghorn, R. A., Ferguson, J. K. W., and Fowler, J. L. A., Changes in body fluids in adrenal insufficiency. Roy. Soc. Canada Proc., 1940, 34, 170.
20. Gregersen, M. I., and Stewart, J. D., Simultaneous determination of the plasma volume with T–1824 and the "available fluid" volume with sodium thiocyanate. Am. J. Physiol., 1939, 125, 142.
21. Winkler, A. W., Elkinton, J. R., and Eisenman, A. J., Comparison of sulfocyanate with radioactive chloride and sodium in the measurement of extracellular fluid. Am. J. Physiol., 1943, 139, 239.
22. Ashworth, C. T., Muirhead, E. E., Thomas, O. F., and Hill, J. M., An analysis of the thiocyanate method for determining the distribution of the body fluids. Am. J. Physiol., 1943, 139, 255.
23. Butler, A. M., and Tuthill, E., An application of the uranyl zinc acetate method for determination of sodium in biological material. J. Biol. Chem., 1931, 93, 171.
24. Shohl, A. T., and Bennett, H. B., A micro method for the determination of potassium as iodoplatinate. J. Biol. Chem., 1928, 78, 643.
25. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry Methods. Baltimore, the Williams and Wilkins Co., 1932, II, 833.
26. Folin, O., and Denis, W., New methods for the determination of total non-protein nitrogen, urea and ammonia in blood. J. Biol. Chem., 1912, 11, 527.
27. Bonnycastle, D. D., and Cleghorn, R. A., A study on the blood volume of a group of untrained normal dogs. Am. J. Physiol., 1942, 137, 380.
28. Ebert, R. V., Stead, E. A., Jr., Warren, J. V., and Watts, W. E., Plasma protein replacement after hemorrhage in dogs with and without shock. Am. J. Physiol., 1942, 136, 299.
29. Noble, R. P., and Gregersen, M. I., Blood volume in clinical shock. I and II. J. Clin. Invest., 1946, 25, 158 and 172.
30. Cleghorn, R. A., Armstrong, J. B., and McKelvey, A. D., A standardized method for producing shock in dogs by bleeding. Canad. M. A. J., 1943, 49, 355 and 363.
31. Cleghorn, R. A., Interim report on clinical and physiological observations on battle casualties. J. Canad. M. Serv., 1945, 2, 145.

EFFECT OF BLOOD PROTEASE AND TRYPSIN INHIBITOR ON THE CLOTTING MECHANISM [1]

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The influence of proteolytic enzymes on blood coagulation has attracted considerable attention in recent years because of the possibility of their involvement in the normal clotting process. Eagle and Harris (1) showed that certain proteolytic enzymes such as trypsin could activate prothrombin; while other enzymes such as papain could clot fibrinogen directly. Schmitz (2, 3, 4) claimed that he isolated both trypsin and a specific trypsin-inhibitor from plasma. However, more recent work indicates that the protease is not identical with trypsin, although it is quite similar in many respects (5, 6).

It has long been known that proteolytic activity appears in blood following treatment with chloroform, acetone, or certain other denaturing agents. Tagnon and others (7, 8, 9) used this technic to activate blood protease and described many of its properties. Holmberg (10) and Christensen (11) independently demonstrated that streptokinase,[2] which is itself devoid of proteolytic activity, could activate the protease in blood and thus produce digestion. Christensen also showed that the blood protease activated by streptokinase was identical with chloroform-activated serum protease (6). The inactive enzyme is located principally in globulin fraction III–2, along with considerable amounts of prothrombin (12, 13). Active protease is present in globulin fraction III–3, which is actually a sub-fraction of III–2. Fraction III–3 was used in the present experiments as a convenient source of blood protease.

A definite coagulation-inhibiting effect of crystalline pancreatic trypsin-inhibitor was demonstrated by Ferguson (14), supporting his thesis that a protease is involved in the activation of prothrombin (15). Similar results were obtained by Grob (16), who also found that serum anti-protease prepared by the method of Schmitz (4) delayed coagulation. Tagnon and Soulier (17) showed that trypsin-inhibitor isolated from soy bean flour could also inhibit coagulation. Their work has been confirmed and extended by the observations described in this paper.

METHODS AND MATERIALS

Most of the experiments described here involve changes in thrombin concentration which can be measured indirectly by means of the clotting time (18). In all cases, solutions were maintained at 38° C., and the pH was kept in the range of 7.2 to 7.6 as measured with a glass electrode. A small amount of phenyl mercuric borate was added to the reagents to prevent bacterial growth. Tests for thrombic activity were carried out by pipetting 0.5 ml. of thrombin into 1 ml. of fibrinogen solution, using 10 mm. × 75 mm. serological tubes. Clot formation was timed from the moment of addition of thrombin or, in the case of plasma, from the moment of recalcification, and the tubes were tilted once each second until the first signs of clotting were observed.

Plasma: Whole blood was collected from dogs under nembutal anaesthesia into paraffined bottles containing 1/10 volume of 3.8 per cent sodium citrate solution. The blood was centrifuged at 1,800 r.p.m. for 30 minutes, and the plasma was siphoned off and re-centrifuged in a high speed angle centrifuge. The plasma was then filtered through a sintered glass disc and kept refrigerated until used.

Buffered saline: M/100 phosphate at pH 7.4 was made up in 0.5 per cent saline.

Fibrinogen: Fibrinogen was prepared by several precipitations with quarter-saturated ammonium sulfate, the final precipitate being dried under vacuum over P_2O_5; 0.15 per cent solutions were made up in buffered saline as needed. The dry powder was stable for over 3 months when kept in the ice box. All fibrinogen solutions were routinely tested for absence of prothrombin by the addition of calcium chloride and thromboplastin.

Calcium chloride: A 1M stock solution of $CaCl_2$ was diluted to M/10 each day as required.

Thromboplastin: Dog brain washed free of blood was macerated in saline, and the extract was preserved by freezing storage. Tests were made for prothrombin by

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[1] This work was supported in part by grants from the Life Insurance Medical Research Fund and the University Center in Georgia.

[2] Following the suggestion of Christensen (11), it was decided to adopt the term "streptokinase" for use throughout this paper. The earlier designation "streptococcal fibrinolysin" was based on the erroneous assumption that the fibrinolytic enzyme itself was produced in the bacterial culture.

the addition of calcium chloride; they were found to be negative.

Prothrombin: The prothrombin was prepared by the acetone precipitation method of Howell (19).

Thrombin: Unless otherwise specified, Lederle's Hemostatic Clotting Globulin diluted 1:10 with buffered saline was used as the source of thrombin.

Soy trypsin-inhibitor: A crystalline preparation of soy trypsin-inhibitor[3] was dissolved in buffered saline to give a solution containing 1 mgm. per ml. This was diluted as required.

Globulin fraction III–3: This alcohol-precipitated fraction[4] contains a protease which is unstable in aqueous solution. Therefore, the dry material was weighed out on an analytical balance and dissolved in saline just before use.

RESULTS

A. *Effect of soy trypsin-inhibitor on the clotting time of recalcified plasma*

The relation of clotting time to concentration of inhibitor was studied by recalcifying citrated plasma in the presence of excess thromboplastin and different quantities of crystalline soy trypsin-inhibitor. One milliliter dilutions of inhibitor in saline were added to 1-ml. portions of plasma, followed by the addition of 0.5 ml. thromboplastin and 0.5 ml. of M/10 calcium chloride. The results presented in Figure 1 show that the increase in clotting time is directly proportional to the concentration of inhibitor in plasma over the range of concentrations used. We have adopted this technic for the rapid assay of inhibitor in various stages of purification by comparing the effect on clotting time with that produced by standard crystalline inhibitor preparations.

B. *Absence of effect of inhibitor on the second phase of coagulation*

Various concentrations of inhibitor were made up by serial dilution in saline and added in 0.5-ml. quantities to 0.5-ml. portions of plasma and fibrinogen solutions. These mixtures were warmed to 38° C. and clotted by the addition of 0.5-ml. thrombin. The clotting times presented in Table I show no inhibitory effect whatsoever, confirming

TABLE 1

Effect of inhibitor on the second phase of coagulation

Inhibitor (micrograms)	0	31	62	125	250	500
Plasma clotting time (seconds)	11″	11″	11″	11″	11″	11″
Fibrinogen clotting time (seconds)	15″	14″	14″	14″	13″	13″

Test mixture: 0.5 ml. plasma + 0.5 ml. inhibitor + 0.5 ml. thrombin.

[3] Crystalline soy trypsin-inhibitor was obtained through the courtesy of Dr. Moses Kunitz.

[4] This protein fraction was obtained through the courtesy of Dr. John T. Edsall. It was prepared under a contract between the Office of Scientific Research and Development and Harvard University from human blood collected by the American Red Cross.

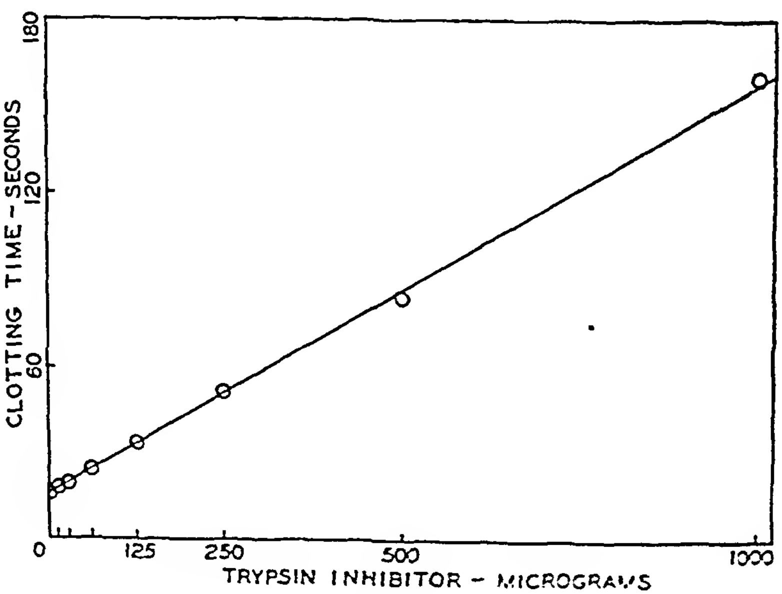

FIG. 1.

the observations of Tagnon and Soulier (17). The absence of inhibition in plasma minimizes the possibility that an albumin co-factor is required for the second-phase action of trypsin-inhibitor (14).

C. *Effect of inhibitor on the activation of prothrombin*

Our attention was next directed to the first phase of coagulation, which normally results in the formation of thrombin. Serial dilutions of inhibitor were made in 1-ml. volumes of buffered saline, and 3 ml. of prothrombin solution was added to each. The mixtures were warmed to 38° C. and activated by the addition of 0.5-ml. quantities of thromboplastin and calcium chloride solutions. After standing for different periods of time, the mixtures were tested for thrombic activity by the addition of 0.5-ml. portions to 1 ml. of fibrinogen solution. The clotting times are presented in Figure 2, from which the following observations are made:

(*a*) The minimum clotting times, indicating maximum concentrations of thrombin, were fairly uniform with low concentrations of inhibitor but showed a marked increase at higher concentrations. The activation time required for maximum thrombin formation was also greater with higher concentrations of inhibitor.

(*b*) The rate of thrombin disappearance increased markedly with higher concentrations of inhibitor. This does not necessarily mean an increased *rate* of thrombinolysis, since Glazko and Ferguson (18) demonstated that a constant rate of thrombin inactivation produced an increase in clotting time which varied inversely with thrombin concentration. The implication is that the rapid increase in clotting time is apparent only because of the small amount of thrombin formed in the presence of inhibitor. An experiment in which 200 µg. of soy inhibitor was added to 1 ml. of thrombin produced no significant changes in thrombic activity over a period of 18 hours at 38° C., showing that the presence of inhibitor *per se* had no influence on the rate of thrombin disappearance.

D. *The effect of the concentration of prothrombin and thromboplastin on the action of inhibitor*

An attempt was made to determine whether the action of trypsin-inhibitor was influenced by variations in the concentration of prothrombin and thromboplastin. Since it has been reported (7) that blood protease is inhibited by prothrombin, especially in the presence of thromboplastin, it was thought that changes in the concentration of these

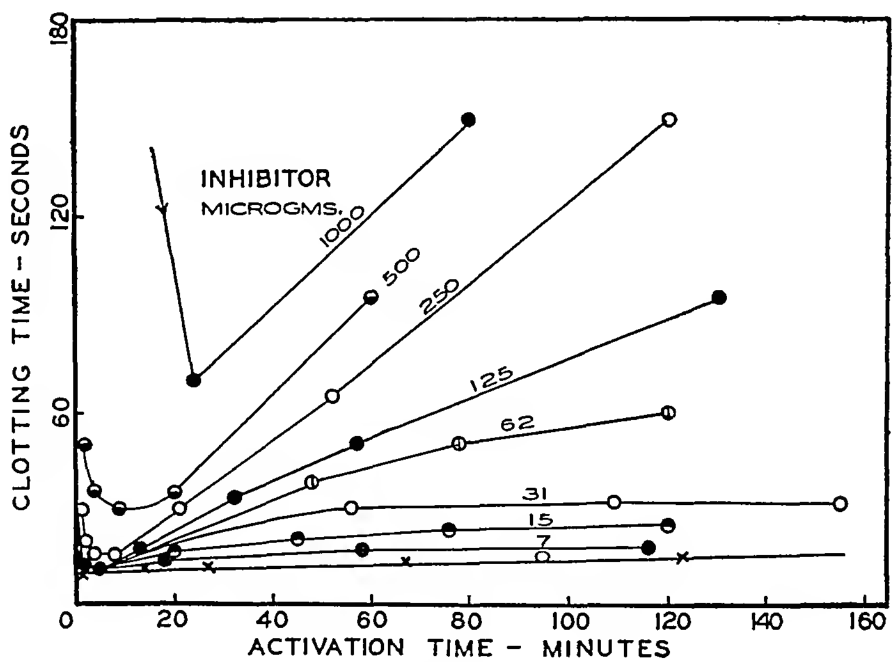

FIG. 2.

factors might produce an exaggerated effect in the presence of trypsin-inhibitor.

The clotting time of recalcified plasma was measured at 38° C. in the presence of soy inhibitor and different concentrations of thromboplastin, as shown in Table II. The range of thromboplastin

TABLE II

Effect of thromboplastin on coagulation of plasma in the presence of inhibitor

Thromboplastin (relative concentration)	Soy trypsin-inhibitor (micrograms)		
	0	25	50
10,000	22″	30″	43″
1,000	21″	25″	37″
100	32″	42″	58″
10	60″	75″	110″
1	170″	270″	510″
0	780″	960″	1380″

Body of table gives clotting times in seconds.
Test mixtures: 0.5 ml. plasma + 0.5 ml. thromboplastin +0.5 ml. inhibitor or saline, recalcified with 0.5 ml. M/10 $CaCl_2$.

concentration extended from that of the stock preparation to a dilution of 1 in 10,000. In all cases, the presence of soy inhibitor produced about the same proportional inhibition with low concentrations as with larger amounts. The results presented in Table III show that sufficient thrombin was produced for reliable clotting tests in a maximum prothrombin dilution of only 1 : 80, under the particular experimental conditions used. The clotting times given are the minimum values observed during the activation process. Here again, the decreased concentration of prothrombin did not appear to enhance the inhibitory effect of trypsin-inhibitor.

TABLE III

Effect of prothrombin concentration on thrombin formation in the presence of inhibitor

Prothrombin (Relative concentration)	Minimum clotting time (seconds)	
	No inhibitor added	125 micrograms inhibitor added
80	15″	21″
8	47″	54″
4	65″	105″
2	85″	240″
1	150″	—

Mixture: 3 ml. prothrombin dilution +1 ml. inhibitor or saline +0.5 ml. thromboplastin +0.5 ml. M/10 $CaCl_2$.

E. *Effect of inhibitor on blood protease*

The effect of trypsin-inhibitor on blood protease was demonstrated by following the rate of casein digestion in the presence of various concentrations of inhibitor. Globulin fraction III–3 was added to 1 per cent casein (at pH 7.4) containing 0, 100, 200 and 400 μg. of soy trypsin-inhibitor. The mixtures were incubated at 38° C. and 1-ml. samples were taken at intervals for determination of acid-soluble phenols, using 6 per cent trichloracetic acid for precipitation of protein and the Heidelberger modification (20) of the Folin-Ciocalteu reaction for color development. From the results presented in Table IV it is evident that the degree of inhibition was proportional to the concentration of soy trypsin-inhibitor in the digestion mixtures. This is in agreement with the results observed with trypsin-inhibitor obtained from pancreas and from serum (4, 6, 16).

TABLE IV

Inhibition of blood protease by soy trypsin inhibitor

Inhibitor (micrograms)	Incubation time (hours)			
	0	1	4	16
0	0	27	43	62
100	0	8	17	30
200	0	3	10	14
400	0	0	7	9
0*	0	0	0	0

* No enzyme added.
Body of table expresses tyrosine equivalent in terms of μg per ml.
Digestion mixture: 10 ml. 1 per cent casein +4 ml. inhibitor dilution +1 ml. (=10 mgm.) globulin III–3 solution.

F. *Thrombin activity of globulin fraction III–3*

Thrombic activity was found to appear in aqueous solutions of globulin fraction III–3 in the absence of any added calcium or thromboplastin, as shown by the data in Table V. This experiment was performed in the presence of sufficient citrate to eliminate the effect of the small amount of calcium present, which was found to be about 2.5 μg. per mgm. of globulin.

It was first thought that the results were due to the direct activation of prothrombin in the globulin fraction by the protease, but similar results were also obtained in the presence of sufficient trypsin-inhibitor to stop the action of the

TABLE V

Thrombin formation by globulin fraction III-3

Incubation time (minutes)	0	1′	2′	3′	5′	10′	30′	70′	330′
Clotting time (seconds)	—	—	44″	40″	39″	34″	32″	35″	42″

10 mgm. globulin was dissolved in 5 ml. saline containing 0.38 per cent sodium citrate. Thrombic activity was measured after different periods of incubation at 38° C. by the addition of 0.5 ml. to 1.0 ml. fibrinogen.

protease. Therefore, it seems likely that the appearance of thrombin in the solution was not due to the activation of prothrombin, but to some other mechanism involving the presence of pre-formed thrombin.

DISCUSSION

The experiments presented here show that crystalline soy trypsin-inhibitor interferes with the activity of the protease in globulin fraction III–3 and also inhibits the activation of prothrombin. There is a good possibility that these observations are dependent on the same process, namely inhibition of proteolytic activity. The activation of prothrombin may be dependent on the presence of active protease, although we have no proof for this as yet. There is good evidence that the protease in blood is not pancreatic trypsin (5, 6), although it is similar in many respects. Tagnon (7) has already indicated that the blood protease can activate prothrombin in the absence of calcium, but his observations have not been confirmed.

Ionic calcium and thromboplastin are unquestionably involved in the normal activation of prothrombin, but their precise functions are still unknown. It is suggested that calcium may be necessary for the activation of blood protease, just as it produces an increased yield of trypsin in the autocatalytic activation of trypsinogen (21). The protease might then function in the activation of prothrombin.

Blood protease and prothrombin are closely associated in many respects. Prothrombin has been reported to inhibit the proteolytic action of chloroform-treated plasma (7). Both prothrombin and inactive protease are present in globulin fraction III–2 (12, 13), while active thrombin and active protease are present in globulin fraction III–3 (see sections E and F). These relations are so striking that they merit further investigation. Some evidence indicates that thrombin itself is proteolytic (22), but the best preparations of thrombin made to date have no demonstrable fibrinolytic activity (23). The ultimate solution of this problem seems to depend on the preparation of pure crystalline thrombin and blood protease.

SUMMARY

1. The anticoagulant action of crystalline soy trypsin-inhibitor is due to interference with the thrombin-forming mechanism. No effects were observed on the second phase of coagulation.

2. The rate of thrombin formation and also the maximum amount of thrombin produced were diminished in the presence of inhibitor.

3. The increase in clotting time of recalcified plasma was found to be directly proportional to the concentration of inhibitor.

BIBLIOGRAPHY

1. Eagle, H., and Harris, T. N., The coagulation of blood by proteolytic enzymes. J. Gen. Physiol., 1937, 20, 543.
2. Schmitz, A., Uber die Proteinase des Fibrins. Ztschr. Physiol. Chem., 1936, 244, 89.
3. Schmitz, A., Uber die freilegung von aktivem Trypsin aus Blutplasma. Ztschr. f. Physiol. Chem., 1937, 250, 37.
4. Schmitz, A., Uber den Trypsin-Inhibitor des Blutes. Ztschr. f. Physiol. Chem., 1938, 255, 234.
5. Kaplan, M. H., Studies on streptococcal fibrinolysis: I. Dissimilarity of serum protease and trypsin as indicated by separate specificities of their kinases, fibrinolysin and enterokinase. J. Clin. Invest., 1946, 25, 331.
6. Christensen, L. R., and MacLeod, C. M., A proteolytic enzyme of serum: characterization, activation, and reaction with inhibitors. J. Gen. Physiol., 1945, 28, 559.
7. Tagnon, H. J., The significance of fibrinolysis in mechanism of coagulation of blood. J. Lab. & Clin. Med., 1942, 27, 1119.
8. Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., Studies on blood coagulation: a proteolytic enzyme prepared from calcium and platelet free normal human blood plasma. J. Clin. Invest., 1942, 21, 525.
9. Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., Studies on blood coagulation: the nature and properties of a proteolytic enzyme derived from plasma. J. Clin. Invest., 1942, 21, 533.

10. Holmberg, C. G., Cleavage of fibrin by streptococcal fibrinolysin. Arkiv Kemi, Mineral., Geol., 1944, **17A**, No. 28.
11. Christensen, L. R., Streptococcal fibrinolysis: A proteolytic reaction due to a serum enzyme activated by streptococcal fibrinolysin. J. Gen. Physiol., 1945, **28**, 363.
12. Edsall, J. T., Ferry, R. M., and Armstrong, S. H., Jr., Clinical, chemical, and immunological studies on the products of human plasma fractionation: XV. The proteins concerned in the blood coagulation mechanism. J. Clin. Invest., 1944, **23**, 557.
13. Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., MacDonald, A. H., and Minot, G. R., Studies in blood coagulation: the coagulation properties of certain globulin fractions of normal human plasma *in vitro*. J. Clin. Invest., 1945, **24**, 698.
14. Ferguson, J. H., Crystalline trypsin-inhibitor and blood clotting. Proc. Soc. Exper. Biol. & Med., 1942, **51**, 373.
15. Ferguson, J. H., A new blood-clotting theory. Science, 1943, **97**, 319.
16. Grob, D., The antiproteolytic activity of serum: II. Physiological significance. The influence of purified trypsin inhibitor on the coagulation of blood. J. Gen. Physiol., 1943, **26**, 423.
17. Tagnon, H. J., and Soulier, J. P., Anticoagulant activity of the trypsin inhibitor from soy bean flour. Proc. Soc. Exper. Biol. & Med., 1946, **61**, 440.
18. Glazko, A. J., and Ferguson, J. H., Kinetics of thrombin inactivation as influenced by physical conditions, trypsin and serum. J. Gen. Physiol., 1940, **24**, 169.
19. Howell, W. H., Prothrombin. Am. J. Physiol., 1914, **35**, 474.
20. Heidelberger, M., and MacPherson, C. F. C., Quantitative micro-estimation of antibodies in the sera of man and other animals. Science, 1943, **97**, 405.
21. MacDonald, M. R., and Kunitz, M., The effect of calcium and other ions on the autocatalytic formation of trypsin from trypsinogen. J. Gen. Physiol., 1941, **25**, 53.
22. Presnell, A. K., Thrombin, a proteolytic fibrinogenase. Am. J. Physiol., 1938, **122**, 596.
23. Seegers, W. H., Purification of prothrombin and thrombin: chemical properties of purified preparations. J. Biol. Chem., 1940, **136**, 103.

A STUDY OF THE DISTRIBUTION AND FATE OF ANTIMONY WHEN USED AS TARTAR EMETIC AND FUADIN IN THE TREATMENT OF AMERICAN SOLDIERS WITH SCHISTOSOMIASIS JAPONICA

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During the Leyte campaign in the Philippines a number of American soldiers were infected by the cercariae of *Schistosoma japonicum.* While under observation at Harmon General Hospital, 138 out of 495 such previously treated patients were found to be passing eggs in their stools and were, therefore, treated again either with tartar emetic or fuadin. The purpose of this report is to present data on the concentrations of antimony attained in body fluids under given dosages of these drugs, together with the amount and rate of excretion in urine and stool. Reference is also made to the therapeutic efficiency of the chemical regimes employed.

MATERIAL AND METHODS

1. *Treatment*

Patients found to have the eggs of *Schistosoma japonicum* in their stools were given in rotation, and without regard to previous treatment overseas, equivalent amounts of antimony (0.566–0.576 gram) in the form of fuadin or tartar emetic (freshly prepared or commercially prepared). One ml. of the fuadin solution contained 0.0087 gram antimony and 1 ml. of tartar emetic solution 0.0018 gram antimony. Fuadin was given by the intramuscular injection of a 6.3 per cent solution, beginning with daily doses of 1.5, 3.5, and 5.0 ml. and continuing with 5.0 ml. doses on alternate days until a total of 65 ml. had been given over a period of 25 days (33 patients). Tartar emetic was given by the intravenous injection of a 0.5 per cent solution on alternate days, beginning with doses of 8, 12, 16, and 24 ml. and continuing with 24-ml. doses until a total of 320 ml. had been given in 29 days (77 patients). Subsequently 15 patients were treated with a course of 105 ml. of fuadin (0.914 gram antimony) and 31 patients with 416 ml. of tartar emetic (0.748 gram antimony). The total number of courses of treatment was 156, but this included some patients with 2 courses of treatment. The times at which various specimens were taken are shown in appropriate tables and figures.

2. *Method of analysis*

The method of Maren (1) has been slightly modified in order to make it more sensitive and specific, and the modified method has been applied to the determination of microgram quantities of antimony in body fluids. Maren states that the blank is of the order of 0.4 microgram and that the iron in blood interferes by giving still larger blanks. The limit of the method in the absence of iron is stated to be 0.5 microgram. When 5-gram specimens of blood are analyzed, he substracts 1 microgram from the final value to compensate for the iron blank. Such a procedure seems inadvisable, especially when the quantity of antimony in the specimen being examined is itself often considerably less than the 1 microgram value being subtracted.

It has been found that the blank may be reduced by the use of chemicals of the proper degree of purity, and it may be eliminated by reading the optical density of the unknown against reagent blanks instead of against benzene. The blank produced by iron has been eliminated by precipitating the iron as ferric-ferrocyanide during the color development. The precision of the method has been further improved by developing the color rapidly at a nearly constant low temperature, by minimizing the fading of the developed color caused by sunlight, and by including specimens of known antimony content along with each lot of unknowns and calculating the unknown values accordingly.

3. *Reagents*

The reagents used were those given by Maren with the following exceptions:

a. Mallinckrodt phosphoric acid was the only one of several brands found to give low consistent blanks.

b. Potassium ferrocyanide 2 grams in 100 ml. of 0.02 per cent Rhodamine B solution.

c. Hydrochloric acid 6 N containing 1 mgm. of ferric iron per ml.

4. *Digestion*

a. Ten ml. or smaller specimens of blood, plasma, urine, or bile are digested according to Maren's directions.

[1] Lt. Colonel, MC.
[2] Major, SnC.
[3] First Lieut., SnC.
[4] Lt. Colonel, MC.

b. Stool: To 5 grams of stool are added 6 ml. of sulfuric acid and 10 ml. of nitric acid. Digestion with nitric acid is carried out in the usual way. Remove the solution from the hot plate, add 2 drops of perchloric acid and immediately put back on the hot plate, in order to eliminate spattering due to the presence of precipitated salts. The solution is cooled after the appearance of copious SO_3 fumes and is diluted with 46 ml. of water giving a 1–10 dilution containing insoluble salts (2 ml. of sulfuric acid are boiled off during the digestion). Place 1 to 5 ml. of this well-mixed dilution into a 125-ml. flask and add 3 ml. of sulfuric acid.

5. *Reduction*

Maren's procedure is used.

6. *Color development*

In order to obtain the maximum color and consistent results, it is essential to develop the color as rapidly as possible at a low temperature and to keep the colored extracts out of direct sunlight. The reagents and glassware used are kept in ice water until used. To the digest flask in an ice water bath, add 5 ml. of 6NHCl and mix. When the mixture is cold, add 2 drops of 0.02 N ceric sulfate and mix. After 2 minutes add 8 ml. of 3 N phosphoric acid and 5 ml. of 0.02 per cent Rhodamine B. Mix and pour all of the mixture into a cold 50-ml. glass-stoppered bottle containing 10 ml. of cold benzene. Shake 200 times in order to extract the red antimony-dye complex. Aspirate off the aqueous layer, decant the benzene layer into a cuvette, and centrifuge about 5 minutes. Since the optical density was proportional to the concentration only when 0 to approximately 5 micrograms of antimony were present, specimens containing more than 5 micrograms were extracted with 20 or 30 ml. of benzene instead of 10 ml., and the results obtained were corrected accordingly.

Because of the small daily variation of color intensity due to varying speeds of color development and room and water temperature, standards were prepared daily by adding known amounts of antimony to normal plasma, blood, et cetera, and carrying these through the entire procedure concurrently with the unknowns. The digestion process was included in the preparation of the standards in order to compensate for possible traces of contaminants in the large amounts of acid used and for the small loss due to spattering during the digestion (probably less than 5 per cent). Reagent blanks and plasma, blood, and other blanks were also prepared in a similar manner.

7. *Note on blood and feces*

The reduced blood digest contains a precipitate of iron salts which interferes in the subsequent color development probably by adsorbing some of the colored complex. This precipitate is dissolved by adding 6 ml. of 4.5 N sulfuric acid and boiling the mixture until SO_3 fumes begin to appear. The yellowish-green solution remains clear and becomes almost colorless on cooling, but precipitation will again occur after several hours. The iron now in solution in the blood digest interferes by giving a blank; so it must be removed in order to obtain precise results. This iron may be removed by precipitation as ferric ferrocyanide, but in the presence of less than 2 mgm. of iron, low recoveries are sometimes obtained, presumably because of adsorption of the colored complex on the semi-colloidal precipitate. In the last 2 sets of determinations, excellent results were obtained by adding enough iron to insure the development of a quickly-flocculating precipitate.

The final changes in the regular procedure when applied to blood are as follows: After reduction the iron salts are first brought into solution. The hydrochloric acid used contains 1 mgm. of ferric iron per ml. (total 5 mgm.). In order to prevent the precipitation of some of the potassium ferrocyanide by the strong acid present in the digest, this reagent is added in combination with the Rhodamine B as a 2 per cent solution. Under these conditions the precipitation is complete and almost instantaneous. Blanks to which have been added as much as 5 mgm. of iron give readings practically identical with those obtained in the absence of iron.

In stools, interference by precipitated salts in the digest was partially eliminated by adding 2 ml. of water after the reduction and heating as with the blood digest. Color was developed in the usual manner with the exception that 12 ml. of phosphoric acid were used to help prevent the precipitation of salts during the process. It is believed that any slight interference by the presence of small amounts of iron can be prevented by following the procedure used with blood.

8. *Calculations*

The optical densities are read in 19 mm. cuvettes on a Coleman Junior Spectrophotometer at a wave length of 565 mu. Reagent blanks, prepared by carrying 5 ml. of water through the entire procedure, usually gave transmittance readings of approximately 98.7 per cent against benzene at 100 per cent. The reagent blanks are set at 100 per cent, and the unknown are read against them. The plasma standards are read against the plasma blanks set at 100 per cent because of the possible presence of traces of antimony in the plasma used. The optical density of the unknown is divided by the optical density per microgram of antimony present in the standard to give the number of micrograms of antimony present in the sample.

9. *Precision of the method*

The precision of the method may be gauged by the data in Table I, in which are recorded the results of recovery experiments done in the last 4 series of determinations of the antimony concentrations of plasma, red blood cells, urine and feces made during a period of over 1 month. The mean error of the plasma recoveries in a range of 1–5 micrograms was 3 per cent. The mean error of the other recoveries was approximately 5 per cent, although the variations encountered on some of these particular days were somewhat greater than usual.

TABLE I

Recovery of added antimony in successive series of determinations

Antimony added	Date	Plasma (5 ml.)	Date	Urine (5 ml.)	Date	Feces (0.5 gram)	Date	Blood (5 ml.)
micrograms		*per cent of average*		*per cent of average*		*per cent of average*		*per cent of average*
	11/15	D = 0.0717	10/31	D = 0.0684	11/6	D = 0.0677	11/24	D = 0.0614
0.5						(1) 103		
1.0		(2) 103 ± 1		(2) 100 ± 0		(1) 98		(2) 91 ± 6
3.0		(2) 99 ± 1		(2) 101 ± 0		(1) 100		(2) 98 ± 2
5.0		(2) 99 ± 2		(2) 99 ± 0		(1) 98		(2) 110 ± 3
	11/16	D = 0.0691	11/7	D = 0.0689	11/9	D = 0.0677	11/27	D = 0.0702
1.0				(2) 102 ± 9		(1) 95		(2) 103 ± 3
3.0		(2) 101 ± 0		(1) 99		(1) 102		(2) 100 ± 0
5.0		(2) 99 ± 4		(2) 99 ± 1		(1) 102		(2) 97 ± 6
	12/1	D = 0.0635	11/20	D = 0.0695	11/19	D = 0.0720	11/22	D = 0.0631
1.0		(1) 93		(2) 109 ± 13		(1) 101		(2) 103 ± 0
3.0		(2) 103 ± 3		(2) 103 ± 3		(1) 99		(2) 97 ± 15
5.0		(2) 100 ± 3		(2) 88 ± 7		(1) 97		(2) 100 ± 3
	12/3	D = 0.0690	11/28	D = 0.0674	12/3	D = 0.0634	12/3	D = 0.0687
1.0		(2) 96 ± 1		(2) 100 ± 2		(2) 90 ± 5		1 ml. (2) 102 ± 3 10 ml. (1) 88
3.0		(2) 107 ± 1		(2) 100 ± 2		(2) 110 ± 1		1 ml. (2) 103 ± 2 10 ml. (2) 103 ± 4
5.0		(2) 97 ± 0		(2) 100 ± 2		(2) 100 ± 3		
		Average error 3 per cent Range −7 per cent to +8 per cent		Average error 4 per cent Range −18 per cent to +22 per cent		Average error 5 per cent Range −15 per cent to +11 per cent		Average error 5 per cent Range −15 per cent to +13 per cent

D = Average optical density per microgram of added antimony.
( ) = Number of determinations.

RESULTS

1. *Plasma concentrations*

Plasma antimony concentrations were determined at various intervals during and after treatment with both tartar emetic and fuadin. The average pre-dose 48-hour values are shown in Figure 1. At the beginning of treatment the average concentration was about 8 micrograms per liter probably as a result of previous courses of treatment. As the course of treatment progressed the plasma concentrations increased rather rapidly to about 85 micrograms per liter after 3 weeks of treatment, and then more slowly to a final concentration of approximately 100 micrograms per liter. The concentrations in the fuadin group increased more rapidly in the early stages of treatment because larger amounts of antimony were given at this time. When the 2 groups of values are compared on the basis of the total amount of antimony administered by each particular time, they are nearly identical. Apparently, equal amounts of antimony given as either tartar emetic or fuadin in approximately equal periods of time result in nearly identical equilibrium plasma antimony concentrations.

After the discontinuance of treatment both groups of plasma concentrations decreased at about the same rate. By 6 days after the final dose (4 days after the 48-hour equilibrium level had been attained) the average concentrations had decreased by about 35 per cent, at 12 days by 52 per cent, and at 28 days by 80 per cent. At 100 days after the end of treatment the average plasma concentration was still 7 micrograms per liter, nearly identical with the pre-treatment value.

The variations of the plasma, blood cell, and urine concentrations at intervals of from 15 minutes to 6 hours after administration of the first dose, the first maximum dose, and 3 other doses including the last one were studied in groups of 4 patients being treated with each drug. The results obtained are shown in Figures 2 and 3. As one would suspect, the maximum plasma concentration was found at the 15-minute interval for tartar emetic and somewhat later for fuadin. The tartar emetic values dropped rather rapidly to nearly the equilibrium levels, but the fuadin plasma concentrations dropped more slowly. The latter values were considerably greater than those of the tartar emetic group at each time interval, although by 4 hours they were approaching the equilibrium values. Apparently, tartar emetic is removed from the plasma more rapidly than is fuadin.

2. *Blood cell concentrations*

At all stages of treatment with both drugs the concentration of antimony in the blood cells was

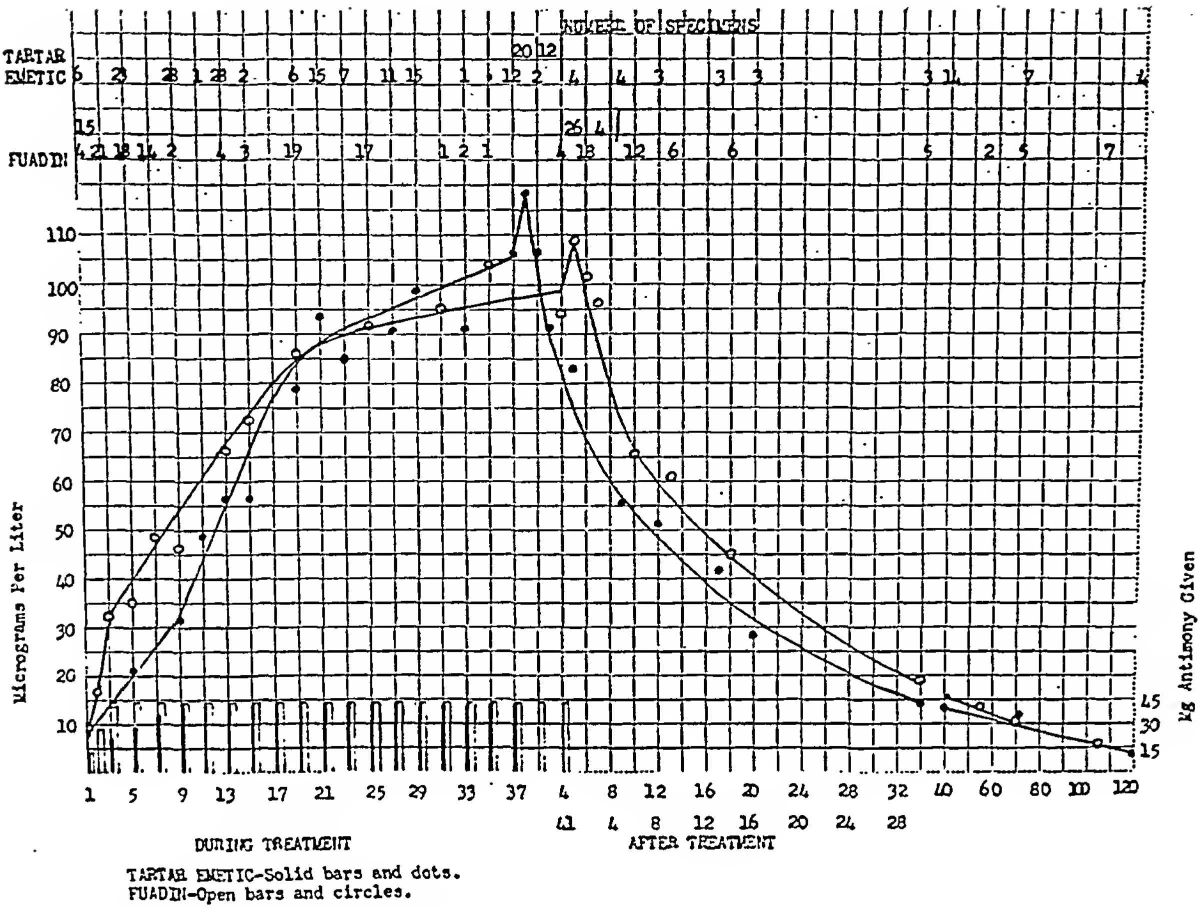


FIG. 1. AVERAGE PRE-DOSE PLASMA ANTIMONY CONCENTRATIONS DURING AND AFTER TREATMENT

definitely greater than that in the corresponding plasma, although the cell-plasma ratios varied during and after the courses of treatment. In the case of tartar emetic the pre-dose cell-plasma ratio decreased from a value of approximately 4 just before treatment to about 1.7 at the completion of treatment. The average pre-dose (48-hour) cell concentrations on various days of treatment were as follows: Day 1, 29; 5, 77; 9, 94; 13, 105; 19, 138; 27, 157; 29, 183; and 37, 195 micrograms per liter. After treatment the cell-plasma ratios again increased with the average cell concentrations being as follows: Day 2, 214; 3, 182; 6–10. 103; 39, 49; 72, 39; and 110, 47.

The pre-dose cell-plasma ratios in the fuadin group varied from about 4 on day 3 of treatment to approximately 1.8 after day 10. The pre-dose cell concentrations on various days were as follows: Day 3, 126; 5, 51; 9, 82; 13, 134; 19, 133; 25, 182; and 41, 165 micrograms per liter. After treatment the ratios again increased to about 4 at 70 days with the average cell concentrations being as follows: Day 1, 215; 2, 186; 3, 206; 6, 131; 35, 68; and 70, 45.

The variations in blood cell concentrations at short intervals after the administration of doses of the drugs were considerably different in the 2 groups. In the tartar emetic group the maximum cell concentrations at 15 minutes were 7–15 times the pre-dose levels and were 6–8 times greater than the plasma concentrations at the same times. The cell concentrations decreased by approximately 50 per cent in the ensuing 45 minutes and more slowly thereafter so that the average cell-plasma ratio at 4 hours was about 10 after the first dose and 3 at the end of treatment.

With fuadin, the cells reached their peak antimony concentrations at about the time of the maximum plasma concentrations. The cell concentrations rose somewhat higher than did those

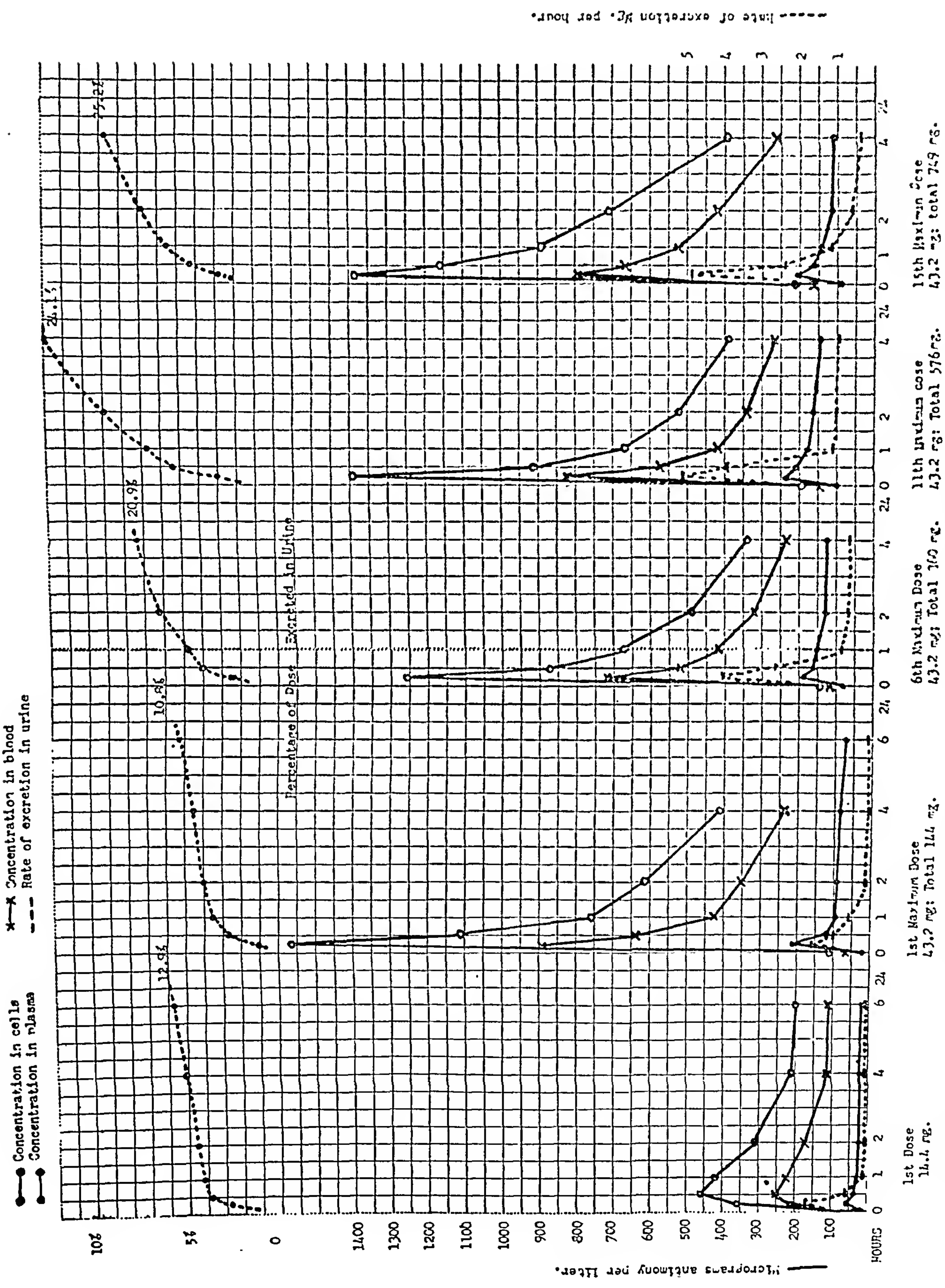


FIG. 2. DISTRIBUTION OF ANTIMONY BETWEEN BLOOD CELLS, PLASMA AND URINE AT INTERVALS AFTER THE ADMINISTRATION OF TARTAR EMETIC

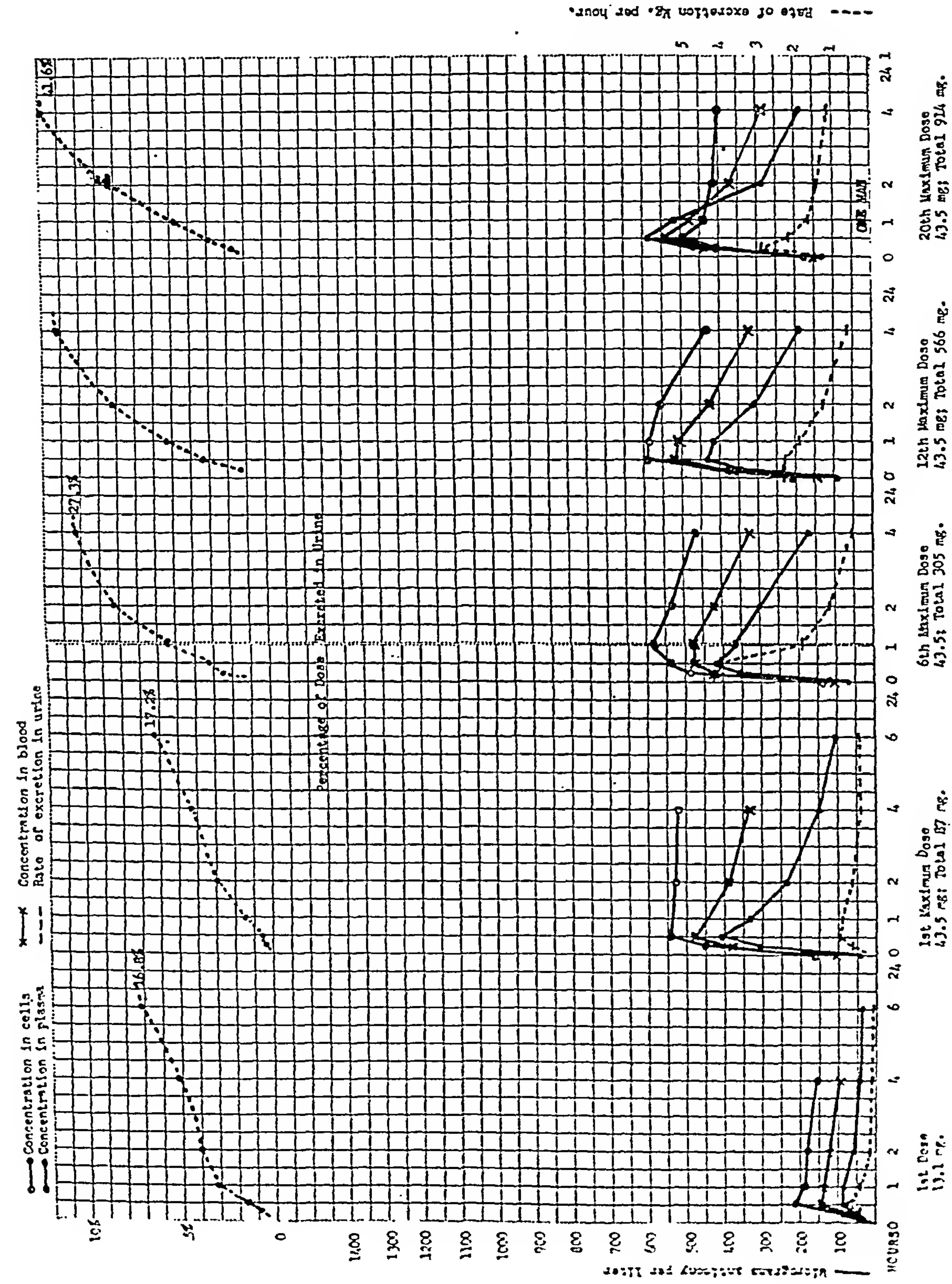


FIG. 3. DISTRIBUTION OF ANTIMONY BETWEEN BLOOD CELLS, PLASMA AND URINE AT INTERVALS AFTER THE ADMINISTRATION OF FUADIN

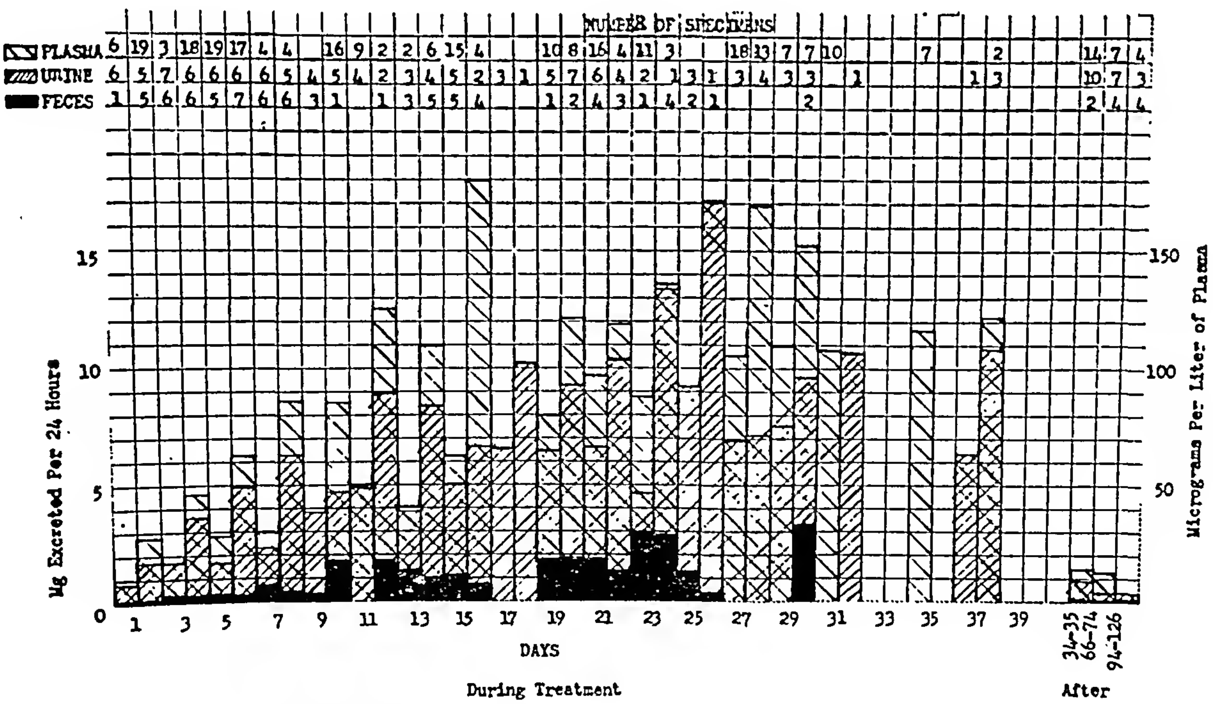
of plasma and they dropped slightly more slowly. The peak concentrations were roughly half as great as those found with tartar emetic and they decreased more slowly so that by 4 hours they were approximately the same as the tartar emetic values. The maximum cell concentrations were 3–4 times the pre-dose levels and were 1–4 times greater than the plasma concentrations at the same times. The average cell-plasma ratio at 4 hours was approximately 3.

Apparently, the early, more rapid decrease of the plasma concentration in the tartar emetic group is at least partially due to a more rapid uptake of tartar emetic by the blood cells. Subsequently this antimony is transferred somewhere else without appreciably affecting the plasma antimony concentration.

3. *Excretion in urine*

In Figures 4 and 5 are plotted the average 24-hour antimony excretions of a few patients at different times during and after treatment. For comparative purposes are shown the average plasma concentrations of larger groups of patients at 2 or at 4 hours after drug administration and also 24 hours later. As is to be expected, the plasma concentration and the urinary excretion on the day of drug administration were greater than on the following day. The excretion increased from nearly 2 mgm. on the day of the first dose to approximately 6 mgm. on the day of the first full dose, to roughly 10 mgm. per day in the latter part of the treatment period. There were no marked differences in the 24-hour excretions of these 2 small groups, although the fuadin values tended to be slightly greater. At approximately 40 days after completion of treatment the average daily excretion was still roughly 1 mgm. per day in both groups while at about 100 days 0.2 mgm. was being excreted per day.

The average urinary antimony excretions of the 4 men in each group studied at short intervals after drug administration are also shown in Figures 2 and 3. In the tartar emetic group the maximum rate of excretion was found in the first 15-minute period at the time of the maximum plasma and blood cell concentrations. The rate rapidly decreased to a fairly constant level by 1 to 2 hours after the dose. The actual urinary antimony concentrations did not follow the same pattern for



Plasma - 2 hours after dose

FIG. 4. COMPARISON OF ANTIMONY EXCRETION WITH PLASMA CONCENTRATION DURING AND AFTER TREATMENT WITH TARTAR EMETIC

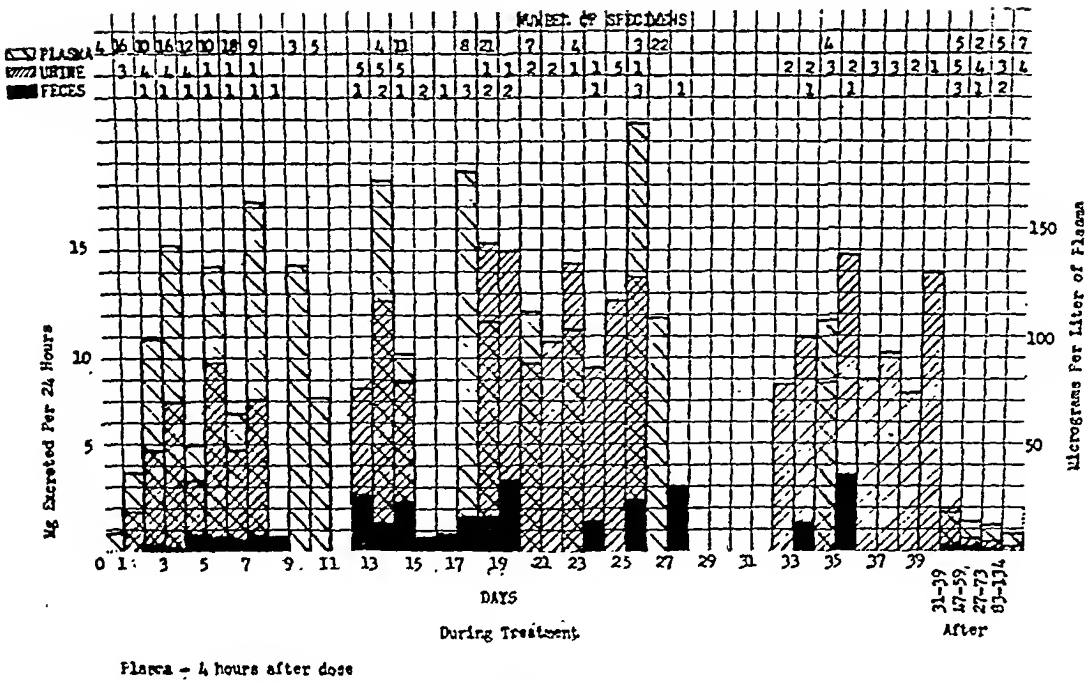


Fig. 5. Comparison of Antimony Excretion with Plasma Concentration During and After Treatment with Fuadin

they tended to be smaller in specimens with larger volumes and lower specific gravities. In a few cases the forcing of fluids before and shortly after drug administration had no marked effect upon the rate of urinary antimony excretion. The average excretion in the first 15 minutes was about 3 per cent of the injected dose; in the first 2 hours it was 6 per cent and the 24-hour excretions ranged from approximately 12 per cent at the beginning of treatment to 25 per cent at the end of treatment.

In the fuadin group the maximum rate of excretion occurred at about the time of the peak plasma concentration. As a rule the maximum rates were lower than those of the tartar emetic group, but the rates of excretion decreased more slowly so that after about 2 hours they were greater than the corresponding tartar emetic rates. The actual urinary antimony concentrations in this group also varied with the specific gravities of the specimens. The average excretion in the first 15 minutes was about 1.3 per cent of the injected dose; in the first 2 hours the excretions increased from about 4 per cent after the first dose to 8 per cent after the last dose. The 24-hour excretions increased from about 17 per cent after the first dose to 27 per cent after the sixth maximum dose, and in one case to 42 per cent after the twentieth maximum dose.

4. *Excretion in feces*

In Figures 4 and 5 are shown the average 24-hour excretions in a limited number of specimens. The average excretion varied from about 0.5 mgm. per day during the first week of treatment to approximately 2 mgm. daily toward the end of treatment. Small amounts were still being excreted at more than 100 days after the completion of treatment.

The combined excretion in urine and feces toward the end of treatment was roughly 24 mgm. in the 2 days following a dose, or approximately 55 per cent of the amount administered in the dose. The remainder presumably was stored in the body.

5. *Concentration in bile*

Interval specimens obtained from 1 patient in each group by duodenal drainage contained definite amounts of antimony. The concentration of antimony increased shortly after drug administration and then slowly decreased.

RESULTS OF TREATMENT

Following the end of a course of treatment 92 of the 138 patients were followed by serial stool examinations (not less than 18 per patient) until the stools again became positive or for a period of 3 months if the stools remained negative. The various technics used on the same specimens consisted principally of the direct smear, gravity sedimentation, and centrifugalization methods. There were 33 patients treated with fuadin and, of these, 27 men, or 82 per cent, were again passing eggs within 3 months or less, after cessation of treatment. These 27 men were again treated with fuadin and 24 of them were again passing eggs in 3 months' time or less. Of the 59 patients treated with tartar emetic only 11, or 19 per cent, were again passing eggs in their stools within 3 months or less.

SUMMARY AND CONCLUSIONS

1. The method of Maren for the determination of microgram quantities of antimony has been modified in order to make it more sensitive and specific. When 0.5- to 5-microgram quantities of antimony were added to 5-ml. portions of plasma, urine, and blood and to 0.5 gram of feces they were recovered with an average error of less than 5 per cent.

2. One hundred thirty-eight patients were given 156 courses of treatment consisting of the administration on alternate days of tartar emetic or of fuadin equivalent to 45 mgm. of antimony. After 0.75 gram of antimony had been given in approximately 5 weeks the average plasma antimony concentration in both groups was about 100 micrograms per liter. Four weeks later the concentration was 20 micrograms per liter. From 15 minutes to 4 hours after drug administration, the fuadin values were considerably greater than those of the tartar emetic group.

3. At all stages of treatment with both drugs the antimony concentration in the blood cells was definitely greater than that of plasma. For several hours after drug administration the tartar emetic group had decidedly higher blood cell concentrations than did the fuadin group.

4. The combined excretion in urine and feces toward the end of treatment was roughly 24 mgm. in 2 days or about 55 per cent of the amount administered in the preceding dose. The remainder presumably was stored in the body. Small amounts were still being excreted more than 100 days later.

5. Ninety-two patients were followed by serial stool examinations for an average of 3 months after completion of treatment. Within this period 82 per cent of 33 patients treated with fuadin were again passing eggs, whereas only 19 per cent of 59 patients treated with tartar emetic were again passing eggs.

6. The question is raised as to whether the higher antimony content in the red blood cells of patients treated with tartar emetic within the first 2 to 4 hours after drug administration than in those treated with fuadin is a factor in the greater therapeutic efficiency of this drug since it is known that the adult worms digest the host's erythrocytes. On the other hand, the known differences of distribution of the two drugs may be directly related to this question.

BIBLIOGRAPHY

1. Maren, T. H., The microdetermination of antimony. Bull. Johns Hopkins Hosp., 1945, 77, 338.

PENICILLIN-RESISTANT STAPHYLOCOCCI: MECHANISMS INVOLVED IN THE DEVELOPMENT OF RESISTANCE

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While staphylococci are among the species of gram-positive bacteria considered to be susceptible to the antibacterial action of penicillin, a number of investigators have reported that there are some strains of coagulase-positive staphylococci which are naturally resistant to penicillin (1 to 11). Of added clinical importance is the fact that pathogenic strains originally sensitive to therapeutic concentrations of penicillin may become resistant to the antibiotic in patients who have received treatment with penicillin (7, 12 to 19). In an attempt to define the nature of the resistance of staphylococci to penicillin, the results of several studies have appeared in which sensitive strains have acquired a high degree of resistance by *in vitro* methods (4, 11, 13, 18, 20 to 24). Todd and Turner (25) succeeded in adapting 2 strains of staphylococcus resistant to penicillin by making repeated subcultures of the cocci in increasing quantities of penicillin. When the strains had acquired resistance, they subcultured the organisms daily in broth without penicillin and observed that there was a rapid decline in resistance. As a result of these observations, they assumed that the resistance of staphylococci which developed in the body was not a permanent characteristic of the organisms and that, upon withdrawal of the drug, the bacteria would quickly revert to a state of sensitivity. On the other hand, Spink and Ferris (26) showed independently that staphylococci which had acquired resistance *in vitro* lost this resistance when grown and transferred in the absence of penicillin, but that penicillin-resistance attained in the body appeared to be a permanent characteristic of the bacteria. This distinction between acquired *in vitro* and *in vivo* resistance has been confirmed by others (11, 18).

One of the major problems related to penicillin-resistance is whether penicillinase plays a role in the resistance displayed by staphylococci. Abraham and Chain (27) first showed that *E. coli* produced an enzyme, designated as penicillinase, which destroyed penicillin, but in a later observation by Abraham and others (4), a strain of *Staph. aureus* adapted by *in vitro* methods to grow in the presence of high concentrations of penicillin did not produce penicillinase. McKee, Rake, and Houck (28) encountered a strain of *Staph. aureus* which was resistant to penicillin and which formed a filter-passing enzyme capable of destroying penicillin. Bondi and Dietz (8) concluded that penicillinase was responsible for the resistance to penicillin of naturally resistant strains of staphylococci, but they (21) also pointed out that other species of bacteria, notably gram-negative organisms, did not owe their resistance to the production of penicillinase. Kirby (29) demonstrated that strains of staphylococcus which were naturally resistant to penicillin possessed a potent inactivator for penicillin, whereas this property was absent in sensitive strains. This observation was extended by Spink and Ferris (26, 30), who showed that strains of staphylococcus made highly resistant to penicillin by *in vitro* adaptation did not produce an inactivator for penicillin; and, in addition, there appeared to be a quantitative relationship between the degree of resistance and the production of an inactivator for penicillin for naturally resistant strains or strains which had acquired resistance *in vivo;* that is, the more resistant a strain, the more potent the inactivator. Demerec (24) has approached the problem of penicillin resistance with a different type of experiment than that used by the foregoing investigators. Starting with a strain of staphylococcus sensitive to the action of penicillin, subcultures were made on agar plates containing penicillin. In this manner, he observed that the sensitive strain gave rise to small numbers of variants which were resistant to low concentrations of penicillin. When these variants were permitted to grow, there was

[1] Aided by grants from Sharp and Dohme, Inc.; the Committee on Scientific Research of the American Medical Association; and the Graduate School, University of Minnesota Medical School.

a gradual increase in variants which were highly resistant to penicillin. Demerec concluded that acquired *in vitro* resistance occurred independently of penicillin and involved a number of mutations and genic changes. In other words, resistance is an inherited characteristic of a given strain, and not acquired through the interaction of the bacteria and penicillin. Although he stated that the resistance persisted for the period of the experiments, it is not known from the data presented whether the resistance was of a permanent nature. Furthermore, it is not known whether the resistant mutants produced an inactivator of penicillin. In a follow-up study of Demerec's work in the same institution, Luria (31) has indicated that there are two types of penicillin-resistance as far as the staphylococcus is concerned, namely, resistance that develops as a result of mutation and resistance that is associated with penicillinase-producing strains. In other words, penicillinase apparently plays no role in the inherited and permanent type of resistance, a conclusion which is at variance with the observations of others and the results in this report. Luria has emphasized that if a small inoculum of organisms is used in the *in vitro* test for sensitivity, penicillinase-producing strains have individual cells which are sensitive to penicillin. However, as will be pointed out shortly, the degree of resistance is related to the *potency* of penicillinase produced by a given strain.

Because there appears to be a lack of general agreement concerning the mechanism or mechanisms whereby penicillin-resistant strains of staphylococcus are established, the present report includes a series of studies which have been carried out over a period of 4 years. Two fundamentally different types of resistance have been observed repeatedly. First, resistance that is acquired *in vitro* by adapting the organisms to grow in increasing concentrations of penicillin. This type of resistance is only a temporary property of the bacteria and is not associated with the formation of penicillinase. The second type of resistance appears to be a permanent characteristic of the strains and is always associated with the production of penicillinase. Furthermore, the degree of resistance is quantitatively related to the potency of penicillinase produced by a given strain. This type of resistance is an inherent property of some naturally resistant strains and of strains which have acquired resistance in the human body as a result of treatment. The methods for obtaining penicillinase from staphylococci and the properties of this penicillinase will be described.

MATERIALS AND METHODS

Strains of staphylococcus

A total of 45 strains of coagulase-positive strains of staphylococcus was studied. All of the strains were recovered from human patients. Of these strains, 25 were sensitive to the action of penicillin; 9 showed a natural type of resistance; and 5 had apparently acquired resistance in the body as a result of treatment.[2] Each of the strains was cultured on veal infusion or tryptose phosphate agar slants and stored in the refrigerator and subcultures were made monthly.

Penicillin

Nineteen lots of commercial sodium penicillin as marketed by 8 different companies were utilized. In the majority of instances, serial and comparative studies of penicillin-resistance and similar studies on the mode of action of penicillinase, were carried out with the same lot of penicillin. It was observed that sodium penicillin could be dissolved in sterile physiological saline, frozen, and then stored in a deep-freeze refrigerator for weeks without loss of potency. Comparative studies were made between commercial sodium penicillin and crystalline sodium penicillin supplied by Merck & Company.

Determination of in vitro *penicillin resistance*

The sensitivity of each strain for penicillin was determined by adding a standard inoculum of organisms to test tubes with liquid medium containing increasing concentrations of penicillin, and then selecting as the end point that tube which showed no turbid growth with the lowest concentration of penicillin. Three types of medium were used for growing the staphylococcus. In the initial experiments, Gladstone's synthetic medium was employed, the preparation of which has been described elsewhere (32). In later studies, veal infusion broth and tryptose phosphate broth were utilized because these media were prepared with less difficulty than Gladstone's medium. The results of the sensitivity tests were essentially the same with all 3 media. The details of determining the *in vitro* sensitivity of each strain to penicillin were as follows: a loopful of culture was taken from an agar slant and grown for 2 to 3 generations in liquid medium. A standard inoculum of 0.1 ml. of 10^{-3} dilution of a 24-hour culture was seeded into each of several test tubes containing one of the foregoing liquid media.

[2] Dr. Donald G. Anderson of Boston kindly supplied us with 3 of the 5 strains which had developed *in vivo* resistance.

Such an inoculum contained between 300,000 to 900,000 organisms. Then 1 ml. of freshly prepared aqueous solution of sodium penicillin was added in ascending concentrations to each of the tubes with the bacterial suspensions. The final volume in each tube was 10 ml. Incubation was carried out for 48 hours at 37° C. and then growth of the organisms was determined by the presence of turbidity. The results of previous studies had emphasized the importance of using a standardized and small inoculum of organisms in performing tests for penicillin sensitivity (5). A large inoculum of organisms required a higher concentration of penicillin for the inhibition of growth than a small inoculum.

Development of in vitro *resistance to penicillin*

Strains of staphylococcus were made resistant to penicillin by exposing succeeding generations of organisms to increasing concentrations of the same lot of commercial sodium penicillin in Gladstone's medium or veal infusion broth. The method was as follows: to each of several tubes containing 9 ml. of liquid medium and 1 ml. of sodium penicillin dissolved in isotonic saline solution was added a standard loopful of a 24-hour culture which had been grown in liquid medium. The initial concentration of penicillin permitted turbid growth when the cocci were incubated for 24 hours at 37° C. When maximum growth was obtained with a given concentration of penicillin after loopful transfers every 24 to 48 hours, the concentration of penicillin was increased and the serial transfers repeated. Of considerable importance was the simultaneous transfer of control organisms from each strain in liquid medium without the presence of penicillin.

Detection of penicillinase production

The presence of crude penicillinase was determined for strains sensitive to the action of penicillin, strains made resistant *in vitro* to penicillin, naturally resistant strains, and strains which had become resistant in the human body. The method of obtaining penicillinase was that of Harper (33) as used for the paracolon bacillus, and as adopted for the staphylococcus by Kirby (34). Essentially, the method consisted of growing each strain on the surface of several agar plates for 24 hours at 37° C., preparing a thick emulsion of the organisms with distilled water, extracting the cocci with acetone and ether, and then drying the bacterial residue *in vacuo*. After testing for sterility, the dried material was stored in the refrigerator.

The potency of the penicillinase was ascertained in the following manner: a standard strain of staphylococcus sensitive to penicillin was grown for 24 hours in Gladstone's medium or broth. Then 0.1 ml. of a 10^{-3} dilution was added to each of several tubes containing synthetic medium or broth with 0.001, 0.01 or 0.1 mgm. per ml. of dried, extracted cells and increasing concentrations of penicillin. The mixtures were incubated at 37° C. for 48 hours and that tube which showed turbid growth with the highest concentration of penicillin indicated the number of units of penicillin destroyed by penicillinase.

RESULTS

I. *Penicillinase from staphylococci*

Kirby (34) first designated the material obtained from the cells of staphylococci which destroyed penicillin as a "penicillin inactivator," and subsequently described some of the properties of this substance (29). Broth suspensions of the inactivator were completely inactivated at 56° C. in 5 minutes. The inactivator could not be obtained in a cell-free filtrate. The rate of destruction of penicillin by the inactivator was the same at 2° C., 22° C., and at 37° C. In contrast to one of these properties, McKee, Rake, and Houck (28) obtained a strain of *Staphylococcus aureus,* appearing as a contaminant in a culture of *Aspergillus flavus,* which produced an enzyme capable of destroying penicillin. This enzyme was obtained in Seitz filtrates of broth cultures. More recently, Luria (31) reported that no inactivator for penicillin was obtained from filtrates of cultures of resistant staphylococci when sintered glass, Seitz, or Mandler filters were used. It appeared desirable to obtain more information concerning the properties of the inactivator of penicillin produced by staphylococci and to study its mode of action. In the following discussion this inactivator will be referred to as penicillinase.

Intracellular nature of staphylococcic penicillinase

It was observed repeatedly that bacterial cells from cultures of staphylococci with the permanent type of resistance contained a potent penicillinase after the cells had been extracted with acetone and ether and then dried. Several attempts were made to obtain the penicillinase in cell-free filtrates by growing the organisms in broth and filtering portions with Berkefeld, fritted glass and Seitz filters. The sterile filtrates contained no active agent against penicillin, thus confirming the findings of Luria (31). Penicillin-resistant organisms were grown in broth for 24 hours, and then the flask shaken vigorously on a shaking machine for 8 hours. When this material was filtered, the sterile filtrate did not contain penicillinase.

Sterile supernatants of penicillin-resistant staphylococci were obtained only after considerable difficulty. Organisms were grown for 24 hours in broth and then the majority of the cells removed

by centrifuging in the usual type of laboratory centrifuge. The supernatant was then centrifuged in a high speed centrifuge (13,000 r.p.m.) in the cold for 1 hour. The top layer of the supernatant was carefully drawn off and this material was centrifuged for another hour. This sterile supernatant did not contain penicillinase.

One hundred milligrams of dried bacterial cells from strain Rosen contained highly active penicillinase. The finely dried powder was added to 100 ml. of tryptose phosphate broth in a flask and the contents shaken vigorously. The powder did not go into solution but was dispensed through the broth as a fine suspension. After equal parts of the broth were filtered through Berkefeld, fritted glass, and Seitz filters, no active penicillinase was present in the filtrates.

The foregoing observations indicate that staphylococcic penicillinase is intimately associated with the bacterial cells and active material could be investigated only with dried cells.

Stability of staphylococcic penicillinase

Sterile dried preparations maintained their potency for several weeks when kept at room temperature or in the refrigerator. When the powder was suspended in sterile physiological saline solution and quickly frozen, there was no diminution in activity. Though no observations were made over an extended period of time, there was no loss of activity of aqueous or broth suspensions kept at room temperature for several days. Penicillinase was found to be heat labile. After 0.01 mgm. per ml. of dried powder prepared from a highly resistant strain was suspended in physiological saline solution and heated at 56° C. for 30 minutes only, 1 unit per ml. of penicillin was destroyed by the heated product, whereas the unheated control inactivated 40 units. After heating at 80° C. for 30 minutes, only slight inactivation of penicillin was noted by the same amount of material.

Effect of pH on action of staphylococcic penicillinase

One mgm. per ml. of a dried preparation from a resistant strain was suspended in physiological saline solution in each of 3 tubes. The pH was adjusted to 2.04, 6.90, and 11.30 and the contents incubated at 37° C. for 30 minutes and for 3 hours. At the end of these periods, the solutions were brought back to neutrality and the activity of the preparations tested against penicillin. The results are tabulated in Table I. It is to be noted that there was a 50 per cent reduction in activity at the end of 30 minutes. In 3 hours, there was complete inactivation at a pH of 11.3 and a 90 per cent reduction in activity at 2.04.

TABLE I

Effect of pH on activity of 1 mgm. per ml. of penicillinase

pH	Per cent reduction in activity after incubation at 37°C.	
	30 minutes	3 hours
2.04	50	90
6.90	0	0
11.30	50	100

In the second experiment, the same concentration of material was incubated at 37° C. for 4 hours at a pH of 3.0, 7.4, and 9.0. Table II shows a 50 per cent reduction of activity on the acid side and complete inactivation in an alkaline medium.

TABLE II

Effect of pH on activity of 1 mgm. per ml. of penicillinase

pH	Per cent reduction in activity after incubation at 37°C. for 4 hours
3.0	50
7.4	0
9.0	100

These results indicate that penicillinase from staphylococcus is more susceptible to changes in hydrogen ion concentration than the penicillinase obtained from an aerobic spore-forming bacillus belonging to the *B. subtilis* group as reported by Woodruff and Foster (35). They observed no change in activity of the enzyme over a range of pH from 3.0 to 11.0. On the other hand, staphylococcic penicillinase appears to be more resistant to changes in hydrogen-ion concentration than the penicillinase studied by McQuarrie and his associates (36) prepared from a gram-negative rod.

Factors influencing the action of staphylococcic penicillinase on penicillin

The inactivation of penicillin by penicillinase was found to be dependent upon 2 main factors. First, the *potency* of the penicillinase utilized in a given

observation, and, second, the period of *time* in which penicillinase was in contact with penicillin. The significance of these 2 factors is illustrated by the following experiments. Preparations of strain Bernardo II in concentrations of 1 mgm. per ml. and 0.1 mgm. per ml. were mixed with 100 units per ml. of penicillin in physiological saline solution and permitted to stand at room temperature for 5 hours. At the end of this period, the penicillinase was removed by filtration and the filtrate tested for penicillin activity. There was only a slight diminution in the potency of penicillin which had been in contact with 0.1 mgm. per ml. of dried cells, whereas there was almost complete destruction of activity with the mixture containing 1 mgm. per ml. When mixtures containing the same quantities of penicillinase and penicillin were allowed to stand for 18 hours at room temperature, there was complete loss of penicillin activity with both preparations.

Pencillinase prepared from strain Rosen always yielded a more potent material than that obtained from strain Bernardo II. When 0.2 mgm. per ml. of Rosen extracted cells were mixed with penicillin and tested for penicillin at the end of 1 and 3 hours, it was observed that there was no loss of penicillin activity in 1 hour, but the potency of penicillin was considerably reduced in 3 hours.

In a preliminary communication (30), this relationship of the potency of penicillinase and the time necessary for inactivation of penicillin was illustrated in another type of experiment. A constant amount of extracted cells was added to each of several tubes containing increasing concentrations of commercial penicillin dissolved in Gladstone's medium. A small inoculum of a penicillin-sensitive strain of staphylococcus was seeded to each of the tubes and the mixtures incubated at 37° C. Growth curves of the organisms were determined by measuring the density of the bacterial cultures with a photoelectric colorimeter at appropriate time intervals. It was shown that with increasing concentrations of penicillin a longer period of time was necessary for inactivation of it. The same type of experiment was repeated in the present study comparing the effect of 0.005 mgm. per ml. of dried Bernardo II cells upon increasing concentrations of commercial penicillin and a purified preparation of crystalline sodium penicillin (Merck). The results are illustrated in Figure 1. The test strain used in this experiment was inhibited in growth by 0.05 unit per ml. of penicillin and

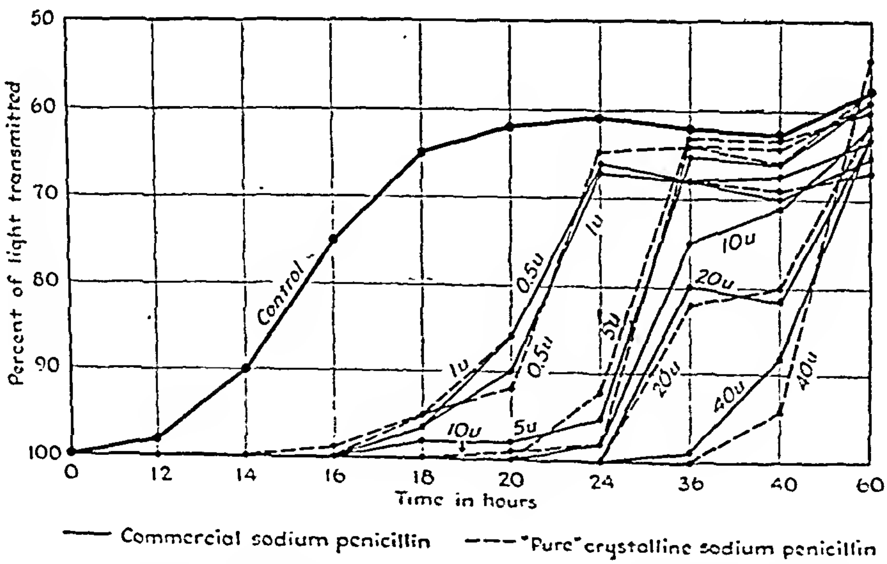

FIG. 1. COMPARATIVE EFFECT OF 0.005 MGM. PER ML. OF STAPHYLOCOCCIC PENICILLINASE ON THE GROWTH CURVES OF A PENICILLIN-SENSITIVE STRAIN OF STAPHYLOCOCCUS IN THE PRESENCE OF INCREASING CONCENTRATIONS OF COMMERCIAL SODIUM PENICILLIN AND "PURE" CRYSTALLINE SODIUM PENICILLIN

Concentrations of penicillin expressed in units per cubic milliliter.

yet, if 0.005 mgm. per ml. of extracted cells containing penicillinase was allowed to remain in contact with penicillin for 40 hours, this strain finally grew out in the presence of 40 units per ml. of penicillin.

Several attempts were made to determine if there was a linear relationship between the inactivation of penicillin by penicillinase. However, no close correlation between these 2 factors was found, probably due in part to the fact that only crude preparations of penicillinase were available. The results of 1 experiment are given in Table III.

TABLE III

Growth of penicillin-resistant strain of staphylococcus in relation to increasing concentrations of penicillin and penicillinase

Incubation at 37°C. for 48 hours

Mgm. per ml. of dried bacterial cells with penicillinase	Units of penicillin per ml.						
	0.05	1	5	10	20	40	60
0.002	+	0	0	0	0	0	0
0.004	+	+	+	+	0	0	0
0.006	+	+	+	+	+	+	0
0.008	+	+	+	+	+	+	+
0.010	+	+	+	+	+	+	+

+ = turbid growth.
0 = no turbid growth.

Each of several tubes contained a standardized small inoculum of a penicillin-sensitive strain of staphylococcus in Gladstone's medium to which were added increasing concentrations of extracted cells and sodium penicillin. The mixtures were incubated for 48 hours at 37° C. and then bacterial growth determined by the presence of turbidity.

Continued action of staphylococcic penicillinase

It was of importance to determine if a preparation of penicillinase would continue to inactivate penicillin after one or more exposures to the antibiotic. This was determined by adding 1 mgm. per ml. of extracted cells to a flask containing tryptose phosphate broth with 100 units per ml. of penicillin. After standing for 24 hours a portion of the mixture was filtered to remove penicillinase, and tested for penicillin activity. Then fresh penicillin was added to the flask and the mixture allowed to stand for another 24 hours, and a filtrate again tested for the presence of penicillin. It was observed that the preparation of penicillinase continued to inactivate penicillin completely until sampling of the mixtures had reduced the concentration of penicillinase to ineffective levels. The contents of a control flask containing only broth and added penicillin did not reveal any loss of penicillin activity when handled in the same manner.

II. Comparison of properties of resistant strains

Staphylococci with resistance acquired by in vitro *methods*

Four strains of staphylococcus isolated from patients were adapted to grow in increasing concentrations of penicillin. Strain Rutgers was cultured from a suppurative skin lesion, while strains Nelson, Ked, and Eden were isolated from patients with osteomyelitis. The initial sensitivity of these strains and the degree of resistance obtained after multiple transfers in Gladstone's medium containing penicillin are shown in Table IV. A significant feature is that the degree of resistance acquired by the strains varied. Strains Rutgers and Nelson required only 6 units per ml. of penicillin to inhibit growth while strains Ked and Eden necessitated 120 and 80 units respectively. It was assumed at the time these observations were made that this acquired penicillin resistance was a relatively permanent property of the strains. Subcultures of these strains were made on veal agar slants and stored in a refrigerator, and transfers were carried out at monthly intervals. At the end of 11 months, the strains were tested for their sensitivity to penicillin and repeated observations revealed that the acquired resistance was not present.

TABLE IV

Development of resistance after multiple transfers in Gladstone's medium with increasing concentrations of penicillin

Strain	No. of transfers in increasing concentrations of penicillin	No. of units per 1 ml. required to inhibit growth	
		Initial sensitivity	After acquisition of penicillin resistance
Rutgers	117	0.05	6
Nelson	117	0.05	6
Ked	115	0.10	120
Eden	113	0.10	80

In order to confirm these observations, the foregoing strains which had acquired and lost their resistance to penicillin were again adapted to

Subculture ... TABLE V

Strains which had acquired resistance to penicillin (Table II) and then had lost this property adapted a second time to grow in the presence of increasing concentrations of penicillin

Strain	No. of transfers in increasing concentrations of penicillin	No. of units per 1 ml. required to inhibit growth	
		Initial sensitivity	After acquisition of penicillin resistance
Rutgers	72	0.05	80
Nelson	72	0.05	40
Ked	72	0.10	600
Eden	72	0.10	20

grow in increasing concentrations of penicillin. This time, these strains became resistant in a shorter period of time and, with the exception of strain Eden, a much greater degree of resistance was attained. The comparative resistance of these strains after 72 transfers is shown in Table V. The strains were then transferred to veal agar slants and placed in the refrigerator for 80 days. At the end of this period, sensitivity tests were carried out and the results revealed no loss of resistance after this one subculture and storage. But after multiple transfers, the resistance was lost.

During the period when the 4 strains were being adapted to penicillin, investigations were made with strain Ked. After 29 transfers in increasing concentrations of penicillin, the culture was plated out on agar, and 10 colonies were picked off the plate and tested for their sensitivity to penicillin. The results are presented in Table VI. It is apparent that in such a culture there are cells possessing different degrees of resistance. This is of

...g III, ...ococci ... noted th... the presence of body fluids, such as hum... ...a, would influence the properties of staphylococci made resistant by *in vitro* methods, strains Ked and Eden were transferred in a medium with penicillin and equal parts of human plasma and veal infusion broth. Although resistance to penicillin was acquired in this manner, the resistance was of only a temporary nature. These results are in agreement with the findings of Blair and his associates (11) who used human serum.

Repeated attempts were made to obtain penicillinase from the cells of those strains with acquired resistance, but in no instance was the material obtained.

In summary, the resistance to penicillin of staphylococci established by *in vitro* methods is characterized by a temporary type of resistance, and the resistance is not associated with the production of penicillinase.

Staphylococci with natural resistance

Nine strains of coagulase-positive staphylococcus were isolated in 1942, or before, from the lesions or blood of patients, subcultured on veal agar slants and stored in the refrigerator. These strains, then, were isolated before penicillin was available for clinical use. In 1943, sensitivity tests were carried out with these strains and it was observed that more than 0.5 unit and less than 1 unit per ml. were required to inhibit growth. These strains were subcultured on agar slants every 2 to

TABLE VI

Sensitivity tests with 10 colonies of strain Ked after 29 transfers in increasing concentrations of penicillin

	Colony number								
	1	2	3	4	5	6	7	8	9
Control without penicillin	+	+	+	+	+	+	+	+	+
Control with 0.05 unit per ml. of penicillin	+	+	+	+	+	+	+	+	+
Control with 0.10 unit per ml. of penicillin	+	+	+	+	+	+	+	+	+
Control with 0.50 unit per ml. of penicillin	+	+	+	+	+	+	+	+	+
Control with 5.00 units per ml. of penicillin	+	+	+	+	+	+	+	+	+
Control with 10.00 units per ml. of penicillin	+	+	+	+	+	+	0	+	+
Control with 20.00 units per ml. of penicillin	+	+	+	+	+	+	0	+	0
Control with 40.00 units per ml. of penicillin	+	0	+	0	0	0	0	0	0
Control with 60.00 units per ml. of penicillin	0	0	0	0	0	0	0	0	0

+ = turbid growth.
0 = no turbid growth.

...sensitivity: penicillinase. ... s observed that a concentration of 0.01 mgm. per ml. of extracted bacteria destroyed 20 to 40 units of penicillin per ml.

Briefly, then, staphylococci possessing a natural resistance for penicillin have 2 properties different from strains which have been adapted by *in vitro* methods to resist penicillin. The resistance appears to be a permanent characteristic of the strains and is associated with the production of penicillinase.

Staphylococci with acquired resistance from patients treated with pencillin

Over a period of 2 years, cultures isolated from 5 patients have been studied extensively. In 4 of the 5 patients staphylococci were obtained before and after treatment with penicillin. Because of the significance attached to these coagulase-positive strains in the present studies, the history of these cultures is presented. Strain Eden was cultured in 1942 from the blood of a young child who had staphylococcic bacteremia and acute osteomyelitis of the tibia. Strain Eden II was obtained from a draining osteomyelitic sinus in the same year after the child had received slightly over 2 million units of penicillin. Strain Long was isolated from a draining lesion in the right costovertebral region originating from a perinephritic abscess. Strain Long II was cultured from the same area after 2 million units of penicillin had been administered. The strains from patients Janson, Bernardo, and Rosen were supplied to us in 1944 through the courtesy of Dr. Donald G. Anderson of Boston. Strain Janson was isolated from an osteomyelitic sinus of a young adult in 1942. After approximately 2 million units of penicillin, strain Janson II was cultured from the same sinus. Strain Bernardo was also isolated from an osteomyelitic lesion at approximately the same time as Janson, and after the administration of 600,000 units of penicillin Bernardo II was cultured from the sinus. It is significant, as Anderson and his associates (14) have pointed out, that cultures of lesions made a year after the completion of treatment with penicillin showed the presence of resistant organisms. Strain Rosen was isolated from the blood lion units of penicillin before the onset of bacteremia. With the exception of Rosen, the comparative increase in resistance of the foregoing strains is given in Table VII.

TABLE VII

Sensitivity of strains of staphylococcus isolated from patients before and after treatment with penicillin

No culture obtained from patient Rosen before treatment

Strain	Units per ml. of penicillin inhibiting growth before treatment	Units per ml. of penicillin inhibiting growth after treatment
Eden	0.10	0.8
Long	0.05	1.0
Janson	0.05	1.0
Bernardo	0.05	2.0
Rosen		20.0

One of the outstanding features of these strains is that the resistance has persisted for up to 4 years, although repeated subcultures have been made. In fact, as will be pointed out shortly, there has been an increase in the degree of resistance. In addition to the characteristic of a persistent resistance, these strains with acquired *in vivo* resistance produce penicillinase. The parent sensitive strains did not yield demonstrable penicillinase.

Attempts to increase resistance of staphylococci with permanent type of resistance by in vitro *methods*

The preceding studies have shown that sensitive strains of staphylococci may be adapted to grow in increasing concentrations of penicillin but that this acquired resistance was only temporary. In view of this, strains with the permanent type of resistance were adapted to grow in increasing amounts of penicillin by methods already described. While these strains were being adapted, simultaneous transfers were made in culture medium without the presence of penicillin.

The first series of observations were made with strain Long II, which had acquired resistance *in vivo* in association with treatment of the patient with penicillin. On 3/3/45, a culture of Long II was divided into 2 parts. Daily transfers were made with one part thereafter in Gladstone's medium, while the other part, known as Long III, was transferred daily in the same medium contain-

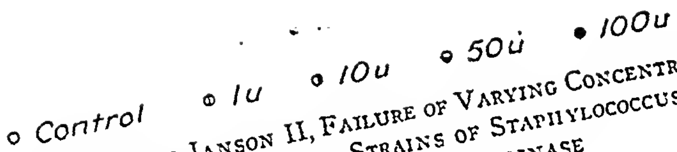

FIG. 3. WITH EXCEPTION OF STRAIN JANSON II, FAILURE OF VARYING CONCENTRATIONS OF PENICILLIN TO INHIBIT GROWTH OF LARGE INOCULUM FROM STRAINS OF STAPHYLOCOCCUS WITH PERMANENT TYPE OF RESISTANCE AND WITH PROPERTY OF PRODUCING PENICILLINASE

ase produced by them are shown in Table IX. iter the 3 strains had acquired further resistance penicillin by methods already described, there ras an associated increase in the potency of penicillinase production as seen in Table IX. Thus, strain Rosen with an acquired resistance which required 400 units of penicillin to inhibit growth produced such a highly active penicillinase that 0.0001 mgm.

TABLE IX

Relationship between the in vitro *resistance of staphylococci and the inactivation of penicillin by penicillinase*

Strain	Strains isolated from patients after treatment with penicillin		Strains isolated from patients after treatment with penicillin and which had acquired further resistance to penicillin *in vitro*	
	No. of units of penicillin per ml. required to inhibit growth	No. of mgm. of dried bacteria which inactivated maximum no. of units of penicillin per ml.	No. of units of penicillin per ml. required to inhibit growth	No. of mgm. of dried bacteria which inactivated maximum no. of units of penicillin per ml.
Long	0.8	0.01 mgm.— 40 units	60	0.001 mgm.— 400 units
Bernardo	2.0	0.01 mgm.—200 units	60	0.001 mgm.— 400 units
Rosen	20.0	0.01 mgm.—600 units	400	0.001 mgm.—1,800 units
				0.0001 mgm.— 400 units

of dried cells inactivated 400 units per ml. of penicillin. The important feature of these observations is that resistance is correlated with penicillinase. The more resistant a strain, the more potent is the penicilli[illegible] produced by that strain. Furthermore, re[illegible] small amounts of penicillinase inactivated large concentrations of penicillin. It should be pointed out, however, that the results as given in Table IX show only a relative quantitative relationship between the degree of resistance and the inactivation of penicillin. This is very probably due to the fact that the method of preparing penicillinase permitted only the use of crude material.

DISCUSSION

These studies emphasize the distinction between the 2 types of resistance to penicillin manifested by staphylococci. A clarification of these differences has been desirable, particularly from the viewpoint of the development of resistant strains of staphylococci in patients as a result of treatment with penicillin. The type of resistance which results from adapting strains *in vitro* to grow in increasing concentrations of penicillin is not a permanent characteristic of these strains, and this resistance is not associated with the production of penicillinase. It is not definitely known whether this type of temporary resistance occurs in the human body following therapy with penicillin. If the phenomenon does occur, it is probably of little clinical significance, since such strains have been shown to be less virulent than the sensitive parent strains (22, 11, 18). The underlying mechanism whereby penicillin-sensitive strains acquire a temporary resistance to the action of penicillin is not clear.

The second type of penicillin resistance, which appears to be a permanent characteristic of the strains and is accompanied by the production of penicillinase by these strains, is of considerable importance in the clinical use of penicillin. The most acceptable explanation for the origin of strains with the permanent type of resistance is that afforded by Demerec (24) and, independently, by Kirby (9), namely, that cultures of penicillin-sensitive strains contain resistant variants capable of mutating toward a state of a high degree of resistance if these cells are given an opportunity to multiply rapidly. The present studies also show that penicillin-resistant strains may acquire a much greater resistance to penicillin if the cells are permitted to multiply rapidly, even in the absence of penicillin.

In describing the development of penicillin-resistant staphylococci by the mechanism of mutation, Demerec (24) did not state whether the resistant cells produced penicillinase. On the other hand, Kirby (29) isolated cells from penicillin-sensitive strains of staphylococci which proved to be resistant to penicillin and which produced penicillinase. In a follow-up study of Demerec's work, Luria (31) discounted the role of penicillinase in the mechanism of penicillin-resistance by emphasizing that the individual cells of resistant and penicillinase-producing strains were sensitive to small concentrations of penicillin. The fundamental question is not whether a given strain produces penicillinase but rather how quickly and how potent is the penicillinase produced by a strain in relation to the concentration of penicillin and the time necessary for the antibiotic to destroy the cells. It is to be recalled that in their original report, Abraham and Chain (27) found that *M. lysodeikticus* produced penicillinase, and yet this strain was sensitive to penicillin. The present investigations show that strains of staphylococci with a relatively high degree of resistance produce a potent inactivator of penicillin; the greater the resistance, the more potent the penicillinase. This relationship of penicillinase production to the resistance of penicillin has been demonstrated for other species of bacteria. Woodruff and Foster (35) described an aerobic spore-forming bacillus belonging to *B. subtilis* group which at pH 6.0 or above was not inhibited in its growth by several hundred units of penicillin because of the penicillinase produced by that strain. However, when the same strain was grown at pH 5.5, no penicillinase was produced, and growth was inhibited by 10 units per ml. of penicillin. McQuarrie and his associates (36), working with 3 strains of spore-forming gram-negative rods, noted a quantitative relationship between penicillin-resistance and the production of penicillinase. The present discussion relates primarily to the mechanism whereby staphylococci resist the action of penicillin because of penicillinase. It is well established that other

mechanisms are responsible for resistance with other species of bacteria since many gram-negative bacteria are highly resistant and yet do not produce penicillinase (21).

The observations presented here and those of others (35 to 39) indicate that the penicillinases from different species of bacteria are not identical but possess different physico-chemical properties, though they all have the common property of inactivating penicillin. It is of interest that Perlstein and Liebmann (40) produced an anti-penicillinase immune serum by injecting rabbits with purified penicillinase. The significance of this observation must await further investigations.

Clinically, the potentiality of penicillin-resistance in staphylococci should be recognized, with the object of treatment being to destroy all the organisms within the shortest possible time. If inadequate concentrations of penicillin are present in the body fluids or tissues of patients, the more susceptible bacterial cells may be eradicated, providing an opportunity for the resistant variants to multiply.

SUMMARY

1. Two types of resistance to penicillin have been described for staphylococci. The first type is an adaptation that may be reproduced *in vitro*, and the resistance is of a temporary nature and not associated with the production of penicillinase.

2. The second type which occurs in patients as a result of treatment with penicillin results in the establishment of strains with a permanent resistance to penicillin, and these strains produce penicillinase.

3. The mechanism whereby temporary resistance takes place is not clearly understood. The permanent type of resistance involves the presence of resistant cells in a penicillin-sensitive strain, which, when permitted to multiply rapidly, establish a uniformly resistant strain.

4. The magnitude of the resistance to penicillin manifested by the permanent type of resistance is quantitatively related to the potency of penicillinase produced by the strains.

5. The establishment of resistant strains of staphylococcus in the human body can be prevented by the prompt use of adequate quantities of penicillin.

BIBLIOGRAPHY

1. Fleming, A., *In vitro* tests of penicillin potency. Lancet, 1942, 1, 732.
2. Hobby, G. L., Meyer, K. L., and Chaffee, E., Activity of penicillin *in vitro*. Proc. Soc. Exper. Biol. and Med., 1942, 50, 277.
3. Schnitzer, R. J., Camagni, L. J., and Buck, M., Resistance of small colony variants (G-forms) of a staphylococcus towards the bacteriostatic activity of penicillin. Proc. Soc. Exper. Biol. and Med., 1943, 53, 75.
4. Abraham, E. P., Chain, E., Fletcher, C. M., Gardiner, A. D., Heatley, N. G., Jennings, M. H., and Florey, H. W., Further observations on penicillin. Lancet, 1941, 2, 177.
5. Spink, W. W., Ferris, V., and Vivino, J. J., Comparative *in vitro* resistance of staphylococci to penicillin and to sodium sulfathiazole. Proc. Soc. Exper. Biol. and Med., 1944, 55, 207.
6. Rantz, L. A., and Kirby, W. M. M., The action of penicillin on the staphylococcus *in vitro*. J. Immunol., 1944, 48, 335.
7. Gallardo, E., Sensitivity of bacteria from infected wounds to penicillin. II. Results in one hundred twelve cases. War Med., 1945, 7, 100.
8. Bondi, A., Jr., and Dietz, C. C., Penicillin resistant staphylococci. Proc. Soc. Exper. Biol. and Med., 1945, 60, 55.
9. Kirby, W. M. M., Bacteriostatic and lytic actions of penicillin on sensitive and resistant staphylococci. J. Clin. Invest., 1945, 24, 165.
10. Meads, M., Ory, E. M., Wilcox, C., and Finland, M., Penicillin sensitivity of strains of six common pathogens and of *Hemophilus hemolyticus*. J. Lab. and Clin. Med., 1945, 30, 725.
11. Blair, J. E., Carr, M., and Buchman, J., The action of penicillin on staphylococci. J. Immunol., 1946, 52, 281.
12. Lyons, C., Penicillin therapy of surgical infections in U. S. Army. J. A. M. A., 1943, 123, 1007.
13. Rammelkamp, C. H., and Maxon, T., Resistance of *Staphylococcus aureus* to the action of penicillin. Proc. Soc. Exper. Biol. and Med., 1942, 51, 386.
14. Anderson, D. G., Howard, L. G., and Rammelkamp, C. H., Penicillin in the treatment of chronic osteomyelitis. Arch. Surg., 1944, 49, 245.
15. Spink, W. W., Hall, W. H., and Ferris, V., Clinical significance of staphylococci with natural or acquired resistance to the sulfonamides and to penicillin. J. A. M. A., 1945, 128, 555.
16. Buchman, J., and Blair, J. E., Penicillin in the treatment of chronic osteomyelitis. Arch. Surg., 1945, 51, 81.
17. Plough, H. H., Penicillin resistance of *Staphylococcus aureus* and its clinical implications. Am. J. Clin. Path., 1945, 15, 446.
18. North, E. A., and Christie, R., Acquired resistance of staphylococci to the action of penicillin. M. J. Australia, 1946, 1, 176.

19. Harley, H. R. S., Baty, J. A., and Bowie, J. H., The pathogenicity of penicillin-insensitive infection. Brit. M. J., 1946, 1, 639.
20. McKee, C. M., and Houck, C. L., Induced resistance to penicillin of cultures of staphylococci, pneumococci and streptococci. Proc. Soc. Exper. Biol. and Med., 1943, 53, 33.
21. Bondi, A., Jr., and Dietz, C. C., Relationship of penicillinase to the action of penicillin. Proc. Soc. Exper. Biol. and Med., 1944, 56, 135.
22. Spink, W. W., Ferris, V., and Vivino, J. J., Antibacterial effect of whole blood upon strains of staphylococci sensitive and resistant to penicillin. Proc. Soc. Exper. Biol. and Med., 1944, 55, 210.
23. Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., Studies on penicillin. II. Observations on therapeutic activity and toxicity. J. Immunol., 1944, 48, 271.
24. Demerec, M., Production of staphylococcus strains resistant to various concentrations of penicillin. Proc. Nat. Acad. Sc., 1945, 31, 16.
25. Todd, E. W., Turner, G. S., and Drew, L. G. W., Temporary character of "fastness" of staphylococci to penicillin. Brit. M. J., 1945, 1, 111.
26. Spink, W. W., and Ferris, V., Penicillin inhibitor from staphylococci which have developed resistance to penicillin in the human body. Proc. Soc. Exper. Biol. and Med., 1945, 59, 188.
27. Abraham, E. P., and Chain, E., An enzyme from bacteria able to destroy penicillin. Nature, 1940, 146, 837.
28. McKee, C. M., Rake, G., and Houck, C. L., Studies on *Aspergillus flavus*. II. The production and properties of a penicillin-like substance—flavacidin. J. Bact., 1944, 47, 187.
29. Kirby, W. M. M., Properties of a penicillin inactivator extracted from penicillin-resistant staphylococci. J. Clin. Invest., 1945, 24, 170.
30. Spink, W. W., and Ferris, V., Quantitative action of penicillin inhibitor from penicillin-resistant strains of staphylococci. Science, 1945, 102, 221.
31. Luria, S. E., A test for penicillin sensitivity and resistance in staphylococcus. Proc. Soc. Exper. Biol. and Med., 1946, 61, 46.
32. Spink, W. W., and Vivino, J. J., Sulfonamide-resistant staphylococci: Correlation of *in vitro* sulfonamide-resistance with sulfonamide therapy. J. Clin. Invest., 1944, 23, 267.
33. Harper, G. J., Inhibition of penicillin in routine culture media. Lancet, 1943, 2, 569.
34. Kirby, W. M. M., Extraction of highly potent penicillin inactivator from penicillin resistant staphylococci. Science, 1944, 99, 452.
35. Woodruff, H. B., and Foster, J. W., Microbiological aspects of penicillin. VII. Bacterial penicillinase. J. Bact., 1945, 49, 7.
36. McQuarrie, E. B., Liebmann, A. J., Kluener, R. G., and Venosa, A. T., Studies on penicillinase. Arch. Biochem., 1944, 5, 307.
37. Duthie, E. S., The production of penicillinase by organisms of the *Subtilis* group. Brit. J. Exper. Path., 1944, 25, 96.
38. Smith, W., and Smith, M., Production of sterile and stable penicillinase. Lancet, 1945, 1, 809.
39. Proom, H., Some observations on penicillinase. Brit. J. Exper. Path., 1945, 26, 98.
40. Perlstein, D., and Liebmann, A. J., The production of anti-penicillinase immune sera. Science, 1945, 102, 197.

PHYSIOLOGIC ACTION OF *CLOSTRIDIUM WELCHII* (TYPE A) TOXINS IN DOGS [1,2,3]

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Recent work reported from this laboratory (1 to 7) has shown that clostridia grow and produce toxins readily in anoxic muscle *in vivo,* and that they are readily introduced into dog muscles during the course of traumatizing procedures. Moreover, our previous experiments have suggested that these and perhaps other bacteria may be responsible for a toxic factor contributing to shock in traumatizing wounds. Since clostridia are known to be frequent contaminants of war wounds (8, 9) it appeared desirable to study the physiologic action of clostridial toxins in order to determine whether these toxins could produce shock [4] alone or in synergy with blood or plasma loss following trauma.

Since the action of these toxins depends on the amount used, the route of injection, and the rate of injection, we have given attention to these variables. Wright (12) has criticized studies on the pharmacologic effects of *Cl. welchii* toxins in which large doses of toxin were administered rapidly, in a manner unlikely to have a parallel in the clinical disease. Study of the effects of the *Cl. welchii* toxic filtrate has a particular interest in that the specific enzymatic nature of its probable principal component (the alpha toxin or lecithinase) (13) and one of its minor components (hyaluronidase) (14) are known. The lethal properties of *Cl. welchii* (Type A) organisms appear to correlate well with their ability to produce the alpha toxin (15). It is therefore possible to interpret physiologic and pathologic effects of these toxins on the basis of their substrate specificities.

MATERIAL AND METHODS

Three preparations of *Cl. welchii* (Type A) toxins have been used: (1) glycerol dialyzed toxic filtrate of *Cl. welchii,* (2) a more purified lecithinase (alpha toxin) from *Cl. welchii,* and (3) a purified *Cl. welchii* hyaluronidase.[5] The glycerol dialyzed toxic filtrate has assayed from 500 to 1,760 mouse subcutaneous MLD (LD_{50}) per ml. in different batches. The Lb (biological limit) varied from 17 to 43 in the various filtrates. It was made by dialyzing sterile filtrates from *Cl. welchii* (Type A), strain BP6K, against glycerol for 18 hours at 4° C. This toxic filtrate has been found by Logan (16) to contain chiefly alpha toxin, a small amount of theta toxin (accounting for fewer than 5 out of a 1,000 MLD), and 100 to 200 viscosity reducing units and 2,000 to 6,000 mucin clot prevention units of hyaluronidase activity per ml. The *Cl. welchii* hyaluronidase preparation contained no demonstrable alpha toxin, 120 van Heyningen units of theta toxin, and greater than 6,400 mucin clot prevention units of hyaluronidase activity per ml. (16). Another enzyme, a decarboxylase, which converts histidine to histamine, frequently present in strains of *Cl. welchii* (17), was not found in the toxic filtrates used in these experiments.

In the majority of experiments, mongrel dogs weighing between 8 and 15 kgm. were fasted overnight, anesthetized intravenously with 30 mgm. of sodium pentobarbital per kgm. of body weight, and were maintained as indicated under light anesthesia by subsequent small doses of the anesthetic. The trachea was exposed and cannulated so that the oxygen consumption could be recorded by a spirometer; the right carotid artery was cannulated and attached to a recording mercury manometer; and the left external jugular vein was exposed so that a catheter could be inserted to the level of the right auricle, in order to obtain mixed venous blood samples and to measure

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[1] This is publication No. 630 of the Cancer Commission of Harvard University, and No. VIII of the series entitled "The Toxic Factors in Experimental Traumatic Shock."

[2] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital.

[3] Grateful acknowledgment is due the Josiah Macy, Jr. Foundation for a grant for the histopathological studies.

[4] We regard shock as a syndrome in which there is primarily a decline in the effective circulating blood volume, followed by a decrease in the minute volume output of the heart, an increase in peripheral vascular resistance, an eventual fall in mean arterial blood pressure and a progressive increase in tissue anoxia, resulting in death. This definition agrees with conceptions commonly held (10, 11).

[5] The first and third have been generously supplied by Dr. Milan A. Logan, and a limited quantity of purified alpha toxin was obtained through the kindness of Dr. A. M. Pappenheimer, Jr.

venous pressure. Femoral vessels were used for obtaining arterial blood and for intravenous injections. In addition to continuous recording of blood pressure, cardiac output was determined at intervals by Fick's principle, and total peripheral vascular resistance was calculated as the quotient: blood pressure/cardiac output. Body temperature was recorded by a rectal thermometer. Heparinized blood samples were taken to measure hemoglobin concentration, hematocrit, and plasma hemoglobin (when hemolysis was present). Hemoglobins were determined on the Evelyn photoelectric colorimeter using the 540 mu filter.

In several cases, electrocardiograms were taken, using the 3 customary leads. In one case, X-ray pictures were taken for cardiac size and configuration. Plasma volume determinations were performed by the T-1824 plasma dye dilution method (18). Plasma protein was estimated by micro-Kjeldahl nitrogen determination. Prothrombin times were done according to the method of Quick (19). All dogs were carefully autopsied and organ weights were recorded. Sections were routinely fixed in Zenker's solution. Certain tissues were fixed in 10 per cent formalin, and frozen sections were stained with Sudan IV for fat.

Cl. welchii toxic filtrate, which was stored in 30 per cent glycerol, was diluted 1:3 with physiologic saline solution before intramuscular injection, and a control experiment was done by using 10 per cent glycerol in saline. Intravenous administration was either "rapid" (the dose being given within 5 minutes) or "slow" (further diluted with saline and given by intravenous drip over a period of 2 to 3 hours). The level of free alpha toxin in the blood stream was followed by means of a modification of the egg yolk turbidimetric method of van Heyningen (20).

In tabulating results, dosages have been expressed in terms of mouse MLD per kgm. body weight. For comparison between the species, it will be recalled that the lethal dose for a mouse should be in the vicinity of 40 to 50 MLD per kgm.

The control dogs, listed in Table I, were treated in precisely the same way as the experimental animals, with the exception that toxic filtrate was omitted. A number of other controls were run, in which either *Cl. welchii* immunized dogs were used, or in which *Cl. welchii* antitoxin was mixed with the toxic filtrate prior to injection into the animal. The experiments on the effect of toxic filtrates on immunized animals will be discussed at length in a future communication (22).

RESULTS

Lecithinase (alpha toxin)

Purified *Cl. welchii* lecithinase (alpha toxin) was given intravenously to 2 dogs, and intramuscularly to 2 dogs. The results of intravenous administration were similar to those which will be described for the glycerol dialyzed perfringens filtrate: a dose of 40 MLD per kgm. caused almost complete hemolysis (terminal hematocrit 5 per cent) and death in 2 hours, with small amounts of hemolyzed pleural and ascitic fluid and massive intestinal bleeding noted at postmortem examination. Intramuscular administration (60 to 90 MLD per kgm.) resulted in only a very small amount of local edema after 5 hours, in contrast to massive swelling produced by the whole perfringens toxin. This could be due to the absence in the purified alpha toxin of some accessory factor, such as hyaluronidase.

Glycerol dialyzed Cl. welchii *(Type A) toxic filtrate*

Intravenous route: Nineteen dogs were given various amounts of *Cl. welchii* toxic filtrate intravenously, ranging from 6 to 800 MLD per kgm. When lethal doses (over 50 MLD per kgm.) were given, a fairly constant physiological picture resulted. Following rapid injection of large doses, the blood pressure fell precipitously to 30 to 40 mm. Hg within 5 to 10 minutes, and then maintained this level for an hour or more before the terminal decline. When smaller amounts of toxic filtrate were given, or when it was given more slowly, there was no immediate response of the blood pressure, but the pressure declined more gradually.

Table I summarizes a number of changes occurring in animals in this group. It will be observed that the cardiac output, blood pressure, hemoglobin, and hematocrit show significant declines prior to death of the animal. There was, however, no significant change in the peripheral vascular resistance. A decrease in the whole blood hemoglobin and hematocrit levels provided the earliest consistent reflection of the downward trend of the animal. Figure 1 illustrates the interrelationships of these data in a typical animal.

In 2 cases, the level of free alpha toxin in the blood stream was followed by van Heyningen's method (20), while toxin was administered by slow intravenous drip. The rise in the level of free alpha toxin in the blood stream was paralleled by a rise in the plasma hemoglobin and a fall in the hematocrit. In 10 out of the 12 dogs receiving a lethal dose of toxin (in which plasma hemoglobin was quantitated), the plasma was deep red, and the plasma hemoglobin reached levels between 1 and 7 grams per cent. Plasma protein concentration

TABLE I

Effects of Cl. welchii *toxic filtrate*

Dog no.	Dose	Wt.	Hours to death	Cardiac output per 10 kgm. dog				Peripheral resistance				Blood pressure				Hemoglobin				Hematocrit			
				Control I	Control II*	1–2 hrs. after toxin	Terminal**	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal
	MLD per kgm.																						
Cl. welchii	I.V.																						
239	805	7.5	1	1.16		.52	.56	200		205	140	130		60	40	10.4	10.3	7.8	5.8	37	37	7	5
313	500	9.6	3	1.27	1.57	.96	1.48	107	76	113	35	126	110	100	48	10.6	10.4	9.8	6.4	39	39	35	24
314	500	12.0	3	3.34	4.00	1.06	.32	28	24	89	175	138	140	136	80	12.1	12.0	13.4	12.0	43	45	48	38
247	324	9.5	1	2.19			.40	55			66	108			24		15.1	12.9	9.2		49	39	20
250	240	8.8	2	1.56		.32	.51	130		244	97	158		60	38		12.0	10.4	5.4		38	35	3
256	240	8.8	3	2.67	1.69	1.44	.59	73	127	148	90	150	168	164	40	12.1	14.6	12.0	6.6		40	40	8
248	228	9.2	2	1.80		.35	.47	93		136	81	142		40	32		12.4	10.4	6.0		40	34	6
296	220	8.7	8	3.16	2.92	4.19	.71	57	62	43	178	138	138	140	96	11.0	10.0	12.6	7.0	38	39	37	19
297	220	11.0	7	2.50	4.51	.65	.50	44	24	116	69	135	128	92	42	14.0	14.1	15.4	7.5	43	43	50	16
Ave. per cent change					+6	−35	−67		−1	+82	+99		−1	−26	−64		+2	−5	−40		+1	−16	−61
P							<.05								<.01				<.05				<.01
Cl. welchii	I.M.																						
240	706	8.5	3¾	.48		.61	.41	302		336	196	105		144	58	15.9	16.3	16.5	14.6	53	61	59	60
243	300	11.6	5½†	2.56		.88	.65	41		58	142	140		145	124	14.8	14.9	16.9	19.8	54	54	54	68
244	300	10.5	9½†	2.47		1.12	.23	50		117	174	135		145	44	13.7	13.3	17.3	25.5	44	46	53	67
241	225	8.0	5	1.21		.95	.34	136		189	121	106		115	26	13.1	13.3	14.8	15.6	46	49	50	57
298	220	8.0	6†	2.36	1.78	.57	.37	97	125	347	308	148	142	128	74	12.7	13.8	15.0	15.4	47	49	52	57
299	220	10.0	6†	2.10	3.60	.73	.22	74	43	164	374	156	154	120	82	14.6	14.6	18.2	21.4	53	53	60	73
300	220	13.2	6½†	.86	.71	.49	.19	94	104	138	296	142	130	118	98		15.5	19.8	21.2		53	72	75
242	167	10.8	6†	2.00		1.30	.87	65		93	123	150		141	125	12.7	14.2	14.2	15.7	42	46	43	50
271	110	10.4	6½†	2.85	1.92		.75	52	82	75	179	158	170	145	145	13.4	14.6	17.8	21.5	47	50	58	70
249	60	10.0	7†	3.63		2.70	.65	40		59	192	144		160	125	12.5	12.4	15.9	18.7	47	45	54	62
270	60	11.5	6†	1.21	2.02	1.65	.52	93	51	107	182	150	138	134	126	14.2	13.8	14.2	15.9	38	45	49	64
Ave. per cent change					−4	−33	−70		+2	+82	+195		−3	−2	−25		+2	+16	+33		+5	+10	+29
P							<.01																<.05

TABLE I—*Continued*

Dog no.	Dose	Wt.	Hours to death	Cardiac output per 10 kgm. dog				Peripheral resistance				Blood pressure				Hemoglobin				Hematocrit			
				Control I	Control II*	1–2 hrs. after toxin	Terminal**	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal
	MLD per kgm.																						
Controls																							
279		9.8	7†	1.68	2.37	1.25	.79	73	45	92	125	116	104	110	94	12.2	13.3	14.6	15.4	43	43	48	51
281		8.0	7†	1.18	1.82	.90	.89	120	67	170	162	90	78	98	92	13.1	15.6	13.8	13.8	41	44	46	43
406		12.3	7½†	3.69		2.58	1.16	28		37	71	160	168	152	124	11.9	10.6	11.1	11.9	40	38	40	39
407		15.5	7¼†	1.71		.98	.81	33		57	68	136	136	134	126	12.6	12.0	13.2	12.4	40	40	47	42
409		11.0	7½†		1.48	2.19	1.08		95	57	88	172	170	152	116	14.4	13.8	16.2	15.4	51	49	56	54
410		12.5	7†		2.08	1.68	1.78		42	54	41	140	138	144	114	13.0	13.4	14.7	16.3	45	48	51	54
411		11.8	7†	1.21	2.11	1.96	1.76	67	39	36	29	114	114	96	70	10.4	10.4	9.6	10.8	37	35	33	26
412		13.4	7†	1.17	2.77	1.65	2.40	54	28	48	34	142	142	138	148	11.7	11.4	13.0	14.2	42	41	45	48
413		12.6	6¾†	.81	.81	.82	1.12	92	74	82	71	120	96	106	124	10.9	10.1	11.4	13.2	38	34	41	47
Ave. per cent change					+43	−21	−29		−36	+47	+61		−4	0	−9		+3	+9	+15		−1	+9	+9

I.V.—Intravenous.
I.M.—Intramuscular.
P—Is the probability that the changes, as compared with the control series, are due to chance (35). Only the statistically significant figures are given.
* Control II was taken 30–60 minutes after Control I.
** Terminal denotes the last value obtained prior to death. Other details given in footnote, Figure 1.
† Killed.

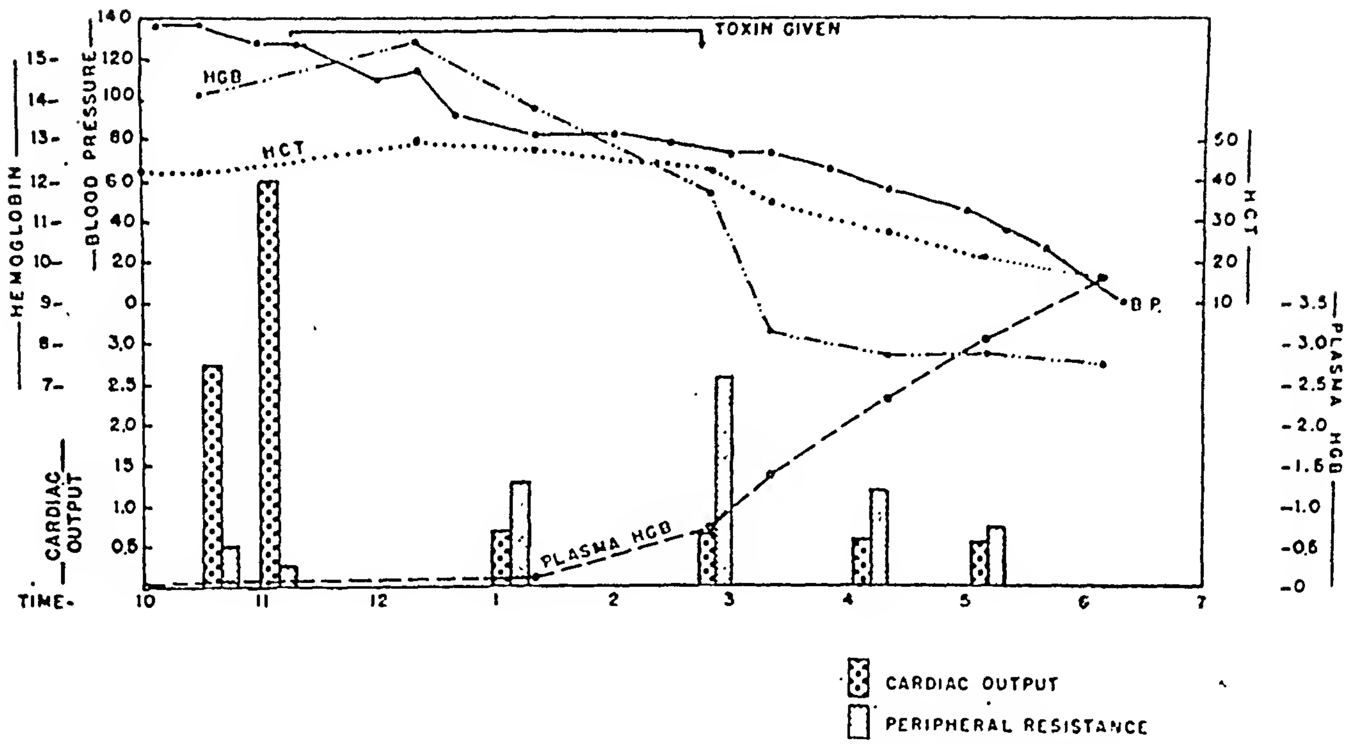

FIG. 1. EFFECT OF *Cl. welchii* TOXIC FILTRATE, ADMINISTERED BY SLOW INTRAVENOUS DRIP

The cardiac output is expressed in liters per minute. The relative peripheral vascular resistance is expressed in arbitrary units as the quotient: blood pressure in mm. mercury/cardiac output in liters per minute. The hemoglobin is expressed as grams per cent.

was measured in 4 such dogs, and (exclusive of hemoglobin) showed no significant change. Plasma volume dye studies were unsatisfactory due to the large amount of hemolysis. No significant change in temperature was observed.

In 4 dogs, electrocardiograms were taken at intervals, after administration of toxic filtrate, up to death 4 to 6 hours later, and failed to show evidence of myocardial damage. X-rays of the heart taken in one animal showed no change in size or configuration as a result of the toxic filtrate. The venous pressure, obtained from the region of the right auricle, was never increased.

Blood clotted poorly following venepuncture in the terminal stages after administration of toxic filtrate. The prothrombin time was tested in 4 dogs given *Cl. welchii* toxic filtrate, with the following results (*cf.* Table II).

Intramuscular route: Twenty-six dogs were injected intramuscularly into one or both triceps surae muscles with *Cl. welchii* toxic filtrate and leg volumes were made on 21 of these animals, according to a technique previously described (4). The most striking effect of the toxins was the development of massive edema, which spread out visibly from the injection site within an hour of the injection and reached a maximum 8 to 16 hours later. By measurement of the increase in volume of the thighs, an estimate of the minimal fluid loss into the limbs was made during a period of 5 to 8 hours after injection. The edema accumulating during this period amounted roughly to 1 to 3 per cent of the body weight, which therefore corresponds to a considerable fraction of the plasma volume. Hemoconcentration was a constant finding, as evidenced by an increase in hematocrit and

TABLE II

Dog no.	Type toxin	Result	Prothrombin time in seconds	
			Before toxin	After toxin
234	alpha	survived (sublethal dose)	13	15
235	alpha	died	13	76
238	glycerol dial. Logan	survived (sublethal dose)	26	81
239	glycerol dial. Logan	died	15	74

a decrease in plasma volume. A consistent fall in cardiac output was present by the sixth hour after injection. The interrelationships of these data are summarized in Tables I and III. It should be pointed out with respect to the intramuscularly injected dogs represented in these tables, that they seldom went into shock in the limited observation period of 6 to 8 hours during which they were under anesthesia and cannulation. The blood pressure (*cf.* Table I) showed no significant change in this group of animals. Two control dogs, injected with 300 MLD per kgm. of toxin mixed with 300 units of antitoxin.[6] showed none of these effects. Only 5 dogs in the series showed any hemolysis, and, in all but 1 case, the hemolysis was under 0.2 gram per cent. Figure 2 illustrates the typical result of this kind of experiment.

TABLE III

Edema-producing effect of Cl. welchii *toxic filtrate following intramuscular injection*

Dog no.	Dose	Elapsed hours to final measurement	5 per cent albumin (B. W.** injected)	Thigh edema: B. W.	Total vascular fluid loss:† B. W.	Fate of animal
	MLD per kgm.		*per cent*	*per cent*	*per cent*	
240	706	3		0.64	1.27	Died*
243	300	5		1.78	2.90	Killed
244	300	8¾		2.02	3.02	Killed
241	225	5		0.80	1.27	Died*
272	220	6½	1.34	1.19	2.33	Killed
273	220	5	3.34	2.10	2.94	Killed
274	220	7½	6.90	2.50	4.62	Killed
286	220	6½	5.55	1.53	3.13	Killed
289	220	6	9.90	2.85	4.34	Killed
290	220	6½	4.45	2.80	3.94	Killed
291	220	5	4.34	1.02	2.87	Killed
298	220	5½		1.69	3.01	Killed
299	220	6		1.09	2.05	Killed
300	220	5¼		1.13	1.67	Killed
278	187	6¾	5.93	2.15	3.15	Killed
372‡	174	6		.14	0.50	Survived
242	167	5½		1.17	2.22	Killed
271	110	6¼		1.60	2.49	Killed
249	60	6½		1.94	2.83	Killed
270	60	5½		.74	1.13	Killed
246	48	8		.89	2.72	Killed
252	12	5½		.97	1.78	Killed
263	4	3		0		Died*

* The lack of correlation between the amount of edema and death of the animal is evident here. It is possible that the toxins reached the general circulation in these instances. Among our control animals an occasional death was attributed to a combination of anesthesia and operative manipulations.
** B. W. signifies body weight.
† Due to blood loss from sampling, plus estimated vascular fluid loss as a result of edema.
‡ Immunized animal (22).

To determine whether the toxic filtrate, when injected intramuscularly, produced general circulatory damage as well as local plasma loss, the following experiments were done:

1. Five dogs injected intramuscularly were treated by administration of 5 per cent bovine albumin[7] in amounts sufficient to maintain normal plasma volume and hematocrit values. Eight control (uninjected) dogs were maintained under similar experimental conditions. Six of these controls were also given albumin to prevent the hemoconcentration which usually occurs in dogs anesthetized with sodium pentobarbital under these conditions. The remaining 2 were untreated.

2. Two intramuscularly injected dogs were allowed to go 5 to 6 hours without treatment; at the end of this period sufficient albumin was given to replace lost plasma and blood. Before the albumin was given, tourniquets were placed around both thighs to prevent further local plasma loss.

3. In 2 dogs, casts (Castex) were placed around both legs extending from the foot pads to the groin. The toxic filtrate was injected through the casts into the triceps surae muscles. Since extensive swelling occurred above the casts (in the regions of the abdominal and gluteal muscles where the amount of swelling could not be quantitated), one was given constant albumin injection, as in (1) and the other was given a terminal albumin injection, as in (2).

The results of these experiments indicated that the decrease in cardiac output which occurred following administration of *Cl. welchii* toxic filtrate intramuscularly could largely be explained by the local loss of plasma into the muscle and subcutaneous tissue in the vicinity of the injection site. Replacement of this plasma loss restored the normal cardiac output temporarily, but did not prevent further plasma loss into the damaged area. In fact, the fluid loss appeared to be somewhat increased by administration of plasma albumin, although the cardiovascular state was better maintained (*cf.* Table III). Edema formation was not prevented by the application of casts, as it extended

[6] Lederle's polyvalent gas gangrene antitoxin.

[7] The authors wish to express their indebtedness to Professor Edwin J. Cohn for generous supplies of purified bovine albumin.

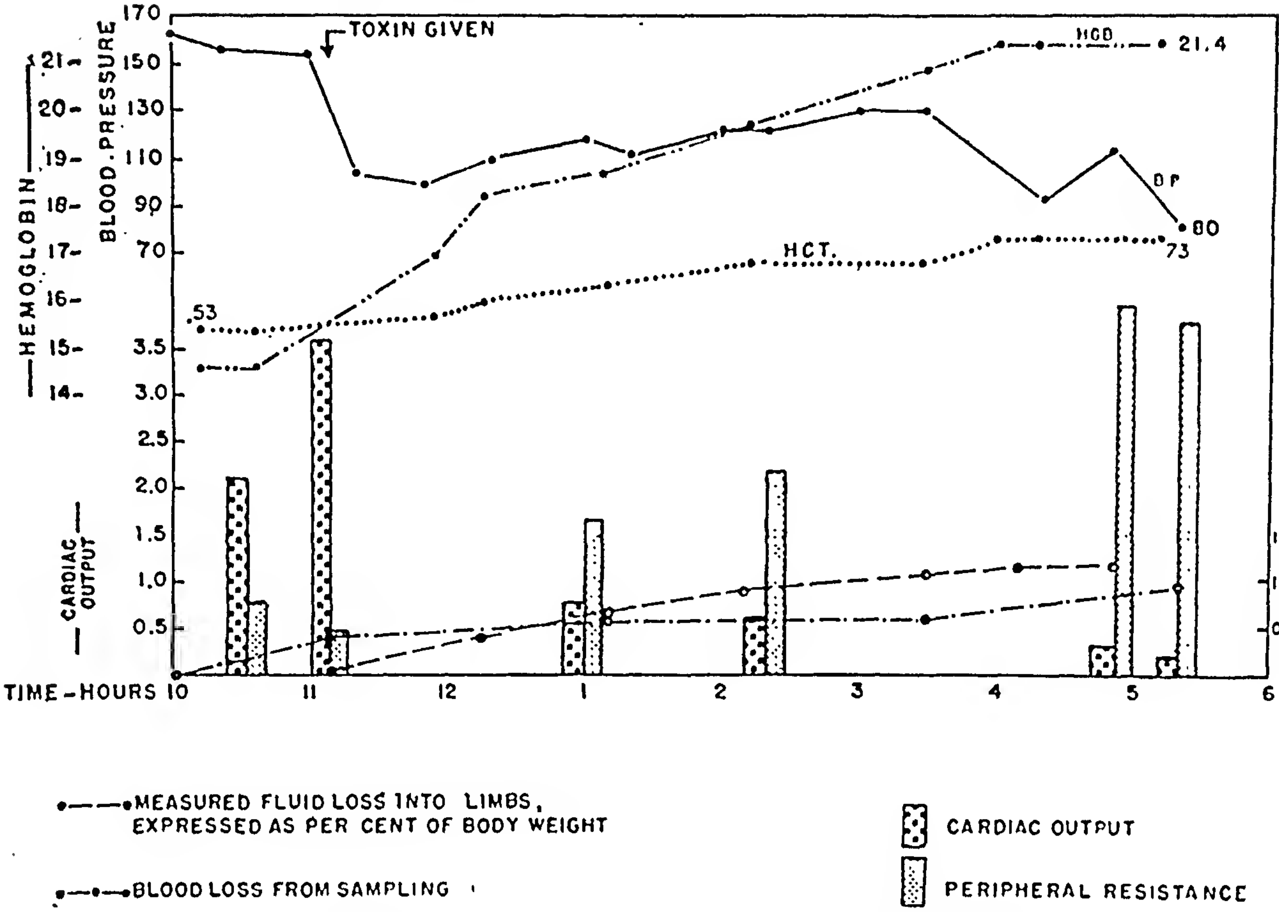

FIG. 2. EFFECT OF *Cl. welchii* TOXIC FILTRATE GIVEN INTRAMUSCULARLY
The blood loss is expressed as per cent of body weight. Other data are expressed as noted in Figure 1.

beyond their confines, occurring in the gluteal, perineal, and retroperitoneal regions if casts were placed about both lower extremities, and toxin was injected through the casts into the underlying musculature. In this case, in a small area immediately adjacent to the injection site, there were muscle necrosis and hemorrhage, but the casted portion of the extremity was edema free.

Three dogs injected subcutaneously with *Cl. welchii* toxic filtrate presented a picture of local edema without detectable generalized effects, similar to the situation seen with the intramuscular injection.

Cl. welchii *hyaluronidase, intramuscular route*

Five-tenths ml. of hyaluronidase was injected into each triceps surae muscle group of an anesthetized dog. During the following 6 hours there was no evidence of generalized effects of the toxin on the cardiac output, peripheral resistance, pulse, respiration, or temperature. There was an area of edema and extravasation of blood 6 cm. in diameter in the region of the injection sites, but no generalized edema of the extremities. Postmortem examination was otherwise completely negative.

Pathologic findings [8]

Cl. welchii toxic filtrate given intravenously in lethal dosage to non-immunized dogs: lungs and liver showed marked congestion and edema. The weights of the lungs were significantly greater than those of control dogs (21). The small intestine, spleen, and kidneys revealed similar findings to a lesser degree. Twenty to 50 ml. of hemolyzed fluid was found in the pleural and peritoneal cavities. Where a similar dosage of toxic filtrate was given to dogs previously immunized there was no congestion of lungs, liver, or other organs. An interesting finding in such animals, however, was the presence of considerable numbers of polymorphonuclear leucocytes in the walls of the pulmonary and adrenal capillaries.

[8] The authors are indebted to Dr. Benjamin Castleman for reviewing and interpreting the pathologic findings.

Cl. welchii toxic filtrate given intramuscularly in lethal dosage to non-immunized dogs: there occurred necrosis and liquefaction of the injected muscle in the vicinity of the injection site, with extensive edema of the surrounding muscle and fascia, and a lesser degree of edema of neighboring muscles. The duodenum showed a marked capillary engorgement. The lungs, liver, adrenal, and spleen were negative. Marked cloudy swelling of the kidney was seen in one animal in this group (No. 240). An excellent, detailed description of the effects of the filtrate on muscle has been given by Robb-Smith (23).

Pathologic findings associated with intravenous injection of *Cl. welchii* toxins have been reported previously (24 to 27). There appears to be some variation among the animal species (dogs, mice, rabbits, guinea pigs) used by the various investigators, in the sensitivity of the erythrocytes to hemolysis and in the localization of the sites of major hemorrhagic manifestations.

DISCUSSION

An attempt has been made to vary the dose, the rate, and the route of administration of *Cl. welchii* toxic filtrate in order to gain a better understanding of its action on the animal. It is impossible to say what quantity of toxic filtrate might be elaborated in gas gangrene or clostridial myositis. It is therefore difficult to transfer information provided by the present experiments to the human problem. It is probable, however, that the closest simulation of clinical conditions is achieved where a relatively small dose of toxic filtrate is administered intramuscularly. Such injections have been demonstrated to evoke vascular fluid loss of considerable proportions. The additive effect of this fluid loss may be sufficient to convert into shock a traumatic condition which would not ordinarily produce this syndrome. Furthermore, the local damage to capillaries resulting from the toxins is long-lasting. If primary shock is averted by adequate therapy, clostridial toxins may introduce the possibility of delayed or secondary shock as a result of continuing fluid loss from damaged capillaries.

The action of the toxic filtrate, administered either intravenously or intramuscularly, is consistent with the damaging effect its lecithinase component might theoretically be expected to have on cell surfaces. In the case of the erythrocyte, the result is rupture of the cell wall; in the case of the vascular endothelium, the result is leakage of whole blood or plasma through a damaged membrane.

It is worth calling attention to the point that either purified *Cl. welchii* alpha toxin or purified *Cl. welchii* hyaluronidase in the absence of the other produced little edema during a 6-hour interval when injected intramuscularly. The edema-forming power of a filtrate containing these 2 toxins (plus a small amount of theta toxin and possibly other unknown toxins) was, however, very great during this time interval. This observation is of interest in connection with the finding of McClean (28) that *Cl. welchii* is able to use hyaluronic acid as a metabolite.

A number of other effects of *Cl. welchii* toxins (Type A) have been described and are discussed in recent reviews on the subject (29, 12). The observation (25) that toxic filtrates of all 4 types of *Cl. welchii* liberate adenyl compounds is especially interesting and may be related to the rupture of cell membranes as a result of the action of the alpha toxin. The recent findings of Cooke *et al.* (9) that fat embolism may occur as a result of *Cl. welchii* myositis suggest that the breakdown products of damaged cells may contribute to the generalized effect of the toxins, even though the toxins themselves may not enter into the general circulation to an appreciable degree.

MacLennan and Macfarlane (30, 31) have been led to consider the possibility "that the profound toxaemia of gas-gangrene is not due solely or primarily to alpha toxin." They point to the ineffectiveness of antitoxin in preventing a lethal outcome once the infection is well established and raise the question as to "whether death is due to the products of tissue breakdown rather than the direct action of bacterial toxin." While the present experiments do not conflict with this interpretation, they offer an additional explanation for the lack of effectiveness of antitoxin in well established cases of gas-gangrene, based on the following 3 points: (*1*) The large amount of fluid lost in clostridial myositis is not repaired by administration of antitoxin. The continuation of fluid loss from damaged blood vessels is likewise

not prevented, since the antitoxin is unable to reverse damage which has already occurred. (2) It has been recently found in this laboratory (32) that once the *Cl. welchii* lecithinase is in contact with its substrate, lecithin, it becomes difficult for antitoxin, when added to the reaction mixture, to prevent the enzymatic process from continuing. It appears that the lecithin and antitoxin compete with each other for the lecithinase. This finding offers a partial explanation for the lack of effectiveness of antitoxin when the disease is well established (*i.e.* when a large amount of lecithinase is in contact with its substrate). (3) It has been found possible to protect mice and dogs from a lethal intravenous dose of *Cl. welchii* filtrate by means of lecithin or total lipids if these are given either in advance of or shortly after the filtrate (33). It is probable that the lecithin protects the animal (by a substrate partition effect) only from the lecithinase component of the filtrate. One may conclude that the lecithinase is in this instance the principal toxic component of the filtrate.

On the other hand, purified alpha toxin produced little edema on intramuscular injection into our dogs. This evidence suggests that collagenase (31), unknown toxins, or a synergistic effect of the combination of toxins plays important roles in the production of edema when a toxic filtrate is given intramuscularly.

An interesting teleological point is raised by the experiments of DeKruif and Bollman (34), who found that the capacity of *Cl. welchii* to invade the animal body was largely dependent on its power to produce toxin. Washed *Cl. welchii* bacilli or spores were infective only in very large numbers; if non-lethal quantities of toxin were added to organisms, only 1/10,000 of the quantity of washed spores or bacilli was necessary to kill the animal. The alpha toxin or lecithinase appears to serve the *Cl. welchii* organism by destroying the surface of animal cells, making the interior available for the nutrition of the *Cl. welchii* organism.

The possibility that effects ascribed to the toxins might be due to substances used in the bacterial culture media has been ruled out by the prevention of these toxic effects both by active immunization of animals and by administration of antitoxin in advance of the toxin (22).

SUMMARY

A number of characteristics of the syndrome of traumatic shock may be produced by *Cl. welchii* (Type A) toxic filtrate given by either intramuscular or intravenous route. Intravenous injection results in intravascular hemolysis, decline in cardiac output and blood pressure, increase in prothrombin time, edema and hemorrhage into the lungs and small intestine, and congestion of the liver, spleen, and kidneys.

Intramuscular or subcutaneous injection is followed by massive edema spreading from the injection site and by severe hemoconcentration. The effects of the toxic filtrate given by this route are chiefly local, and the lack of hemolysis is evidence of the inability of the alpha toxin to reach the general circulation in appreciable concentration.

Purified *Cl. welchii* alpha toxin injected intramuscularly produced little edema. Purified *Cl. welchii* hyaluronidase similarly had little effect on intramuscular injection. The edema-forming property of the unpurified *Cl. welchii* toxic filtrate thus appears to be due to the presence of other toxins or to a synergistic effect of a combination of toxins.

Cl. welchii toxins may play an important role in certain cases of traumatic shock by aggravating fluid loss into the injured region.

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BIBLIOGRAPHY

1. Aub, J. C., Brues, A. M., Dubos, R., Kety, S. S., Nathanson, I. T., Pope, A., and Zamecnik, P. C., Bacteria and the "toxic factor" in shock. War Med., 1944, 5, 71.
2. Nathanson, I. T., Nutt, A. L., Pope, A., Zamecnik, P. C., Aub, J. C., Brues, A. M., and Kety, S. S., The toxic factors in experimental traumatic shock. I. Physiologic effects of muscle ligation in the dog. J. Clin. Invest., 1945, 24, 829.
3. Brues, A. M., Cohn, W. E., Kety, S. S., Nathanson, I. T., Nutt, A. L., Tibbetts, D. M., Zamecnik, P. C., and Aub, J. C., The toxic factors in experimental traumatic shock. II. Studies on electrolyte and water balance in shock. J. Clin. Invest., 1945, 24, 835.
4. Kety, S. S., Nathanson, I. T., Nutt, A. L., Pope, A., Zamecnik, P. C., Aub, J. C., and Brues, A. M.,

The toxic factors in experimental traumatic shock. III. Shock accompanying muscle ischemia and loss of vascular fluid. J. Clin. Invest., 1945, 24, 839.
5. Aub, J. C., Brues, A. M., Kety, S. S., Nathanson, I. T., Nutt, A. L., Pope, A., and Zamecnik, P. C., The toxic factors in experimental traumatic shock. IV. The effects of the intravenous injection of the effusion from ischemic muscle. J. Clin. Invest., 1945, 24, 845.
6. Zamecnik, P. C., Aub., J. C., Brues, A. M., Kety, S. S., Nathanson, I. T., Nutt, A. L., and Pope, A., The toxic factors in experimental traumatic shock. V. Chemical and enzymatic properties of muscle exudate. J. Clin. Invest., 1945, 24, 850.
7. Pope, A., Zamecnik, P. C., Aub, J. C., Brues, A. M., Dubos, R. J., Nathanson, I. T., and Nutt, A. L., The toxic factors in experimental traumatic shock. VI. The toxic influence of the bacterial flora, particularly *Clostridium welchii*, in exudates of ischemic muscle. J. Clin. Invest., 1945, 24, 856.
8. MacLennan, J. D., Anaerobic infections of war wounds in the Middle East. Lancet, 1943, 2, 63, 94, 123.
9. Cooke, W. T., Frazer, A. C., Peeney, A. L. P., Govan, A. D. T., Barling, S. G., Thomas, G., Leather, J. B., Elkes, J. J., and Scott-Mason, R. P., Clostridial infections in war wounds. Lancet, 1945, 1, 487.
10. Blalock, A., Mechanism and treatment of experimental shock. I. Shock following hemorrhage. Arch. Surg., 1927, 15, 762.
11. Wiggers, C. J., The present status of the shock problem. Physiol. Rev., 1942, 22, 74.
12. Wright, G. P., Toxic factors in wound infection. Bull. War. Med., 1942, 2, 251.
13. Macfarlane, M. G., and Knight, B. C. J. G., The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. Biochem. J., 1941, 35, 884.
14. Chain, E., and Duthie, E. S., Identity of hyaluronidase and spreading factor. Brit. J. Exper. Path., 1940, 21, 324.
15. Evans, D. G., The *in vitro* production of α toxin, θ haemolysin and hyaluronidase by strains of *Cl. welchii* type A, and the relationship of *in vitro* properties to virulence for guinea pigs. J. Path. and Bact., 1945, 57, 75.
16. Logan, M. A., Personal communication.
17. Gale, E. F., Production of amines by bacteria. 4. The decarboxylation of amino acids by organisms of the groups *Clostridium* and *Proteus*. Biochem. J., 1941, 35, 66.
18. Gibson, J. G., 2nd., and Evans, W. A., Jr., Clinical studies of the blood volume. 1. Clinical application of a method employing the azo dye "Evans Blue" and the spectrophotometer. J. Clin. Invest., 1937, 16, 301.
19. Quick, A. J., The Hemorrhagic Diseases and the Physiology of Hemostasis. Charles C. Thomas, Springfield, Ill., 1942, 312.
20. van Heyningen, W. E., The biochemistry of the gas gangrene toxins. I. Estimation of the α toxin of *Cl. welchii* type A. Biochem. J., 1941, 35, 1246.
21. Aub, J. C., Zamecnik, P. C., and Nathanson, I. T., Physiologic action of *Clostridium oedematiens* (Novyi) toxin in dogs. J. Clin. Invest., 1947, 26, 404.
22. Nathanson, I. T., Rubin, J., Aub, J. C., and Zamecnik, P. C., Unpublished data.
23. Robb-Smith, A. H. T., Tissue changes induced by *Cl. welchii* type A filtrates. Lancet, 1945, 2, 362.
24. Görtzen, J., Histologische Veränderungen bei kleinen Versuchstieren nach intravenöser Einführung des Toxins des Fraenkelschen Gasbazillus (*B. Welchii*, *B. perfringens*). Zentralbl. f. Bakt. (Abteilung 1, Originale), 1936, 138, 366.
25. Weinberg, M., and Combiesco, N., Lésions provoquées dans l'organisme animal par l'injection intraveineuse de toxine du Bacillus perfringens (hémoglobinurie, pigmentation, hémorragies, nécroses). Compt. rend. Soc. de Biol., 1930, 103, 1091.
26. Kellaway, C. H., and Trethewie, E. R., The injury of tissue cells and the liberation of pharmacologically active substances by the toxins of *Cl. welchii*, types B and C. Australian J. Exper. Biol. and M. Sc., 1941, 19, 77.
27. Favata, B. V., Dowdy, A. H., Sewell, R. L., and Vincent, J. G., The pathology of experimental clostridial infections in dogs. Surg., Gynec., Obst., 1944, 79, 660.
28. McClean, D., and Hale, C. W., Studies on diffusing factors. The hyaluronidase activity of testicular extracts, bacterial culture filtrates and other agents that increase tissue permeability. Biochem. J., 1941, 35, 159.
29. Oakley, C. L., The toxins of *Cl. welchii*; critical review. Bull. Hyg., 1943, 18, 781.
30. MacLennan, J. D., and Macfarlane, R. G., Toxin and antitoxin studies of gas-gangrene in man. Lancet, 1945, 2, 301.
31. Macfarlane, R. G., and MacLennan, J. D., The toxaemia of gas-gangrene. Lancet, 1945, 2, 328.
32. Zamecnik, P. C., and Lipmann, F., A study of the competition of lecithin and antitoxin for *Cl. welchii* lecithinase. J. Exper. Med., 1947, 85, 395.
33. Zamecnik, P. C., Folch, J., and Brewster, L., Protection of animals against *Cl. welchii* (Type A) toxin by injection of certain purified lipids. Proc. Soc. Exper. Biol. and Med., 1945, 60, 33.
34. DeKruif, P. H., and Bollman, J. L., The toxin of *B. welchii*, II. The mechanism of infection with *B. welchii*. J. Infect. Dis., 1917, 21, 588.
35. Arkin, H., and Colton, R. R., An Outline of Statistical Methods, 4th edition. De Pamphilis Press, Inc., New York, 1939, p. 127.

TABLE I

Effects of Cl. oedematiens *toxin administered intravenously*

Dog no.	Dose	Wt.	Hours to death	Cardiac output per 10 kgm. dog				Peripheral resistance				Blood pressure				Hemoglobin				Hematocrit			
				Control I	Control II*	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal	Control I	Control II	1–2 hrs. after toxin	Terminal
	MLD per kgm.																						
335	3,750	11.2	4	1.16	.88	.36	.18	105	132	178	232	154	146	80	52	10.0				39		35	
259	2,525	9.9	3¼	1.36	1.43	.43	.18	83	80	159	191	110	110	66	34	10.7	11.3	12.6	15.4	41	41	41	58
301	2,500	10.0	2	5.25	2.44	.37		24	52	135		130	127	50		11.7	11.3	11.7	13.8	40	40	39	49
302	2,500	8.5	5¼	4.06	2.39	.52	.47	51	79	332	228	148	136	116	78	13.1	13.3	13.7	15.0	48	44	50	44
303	2,500	10.0	3	6.18	2.60	.66	.24	23	54	218	284	134	140	144	68	14.5	15.6	14.8	20.3	50	44	53	71
304	2,500	10.0	3¾	2.19	2.96	.97	.38	57	41	144	169	124	120	140	64	11.1	10.8	11.9	14.8	44	43	47	59
337	2,500	11.7	3	1.07	2.56			100	40			146	140	94	46	13.1	13.1	14.1		46	50	51	
264	2,075	12.0	4	1.96	1.45	.76	.17	52	73	126	113	148	152	138	30	14.8	16.9	15.9	15.9	49	51	53	56
380	1,760	14.5	4	2.79	1.39	.52	.18	21	43	116	143	126	128	128	54	13.4	12.8	13.8	16.7	46	46	50	68
305	1,500	10.0	4¾	.72	2.58	.64	.20	164	54	195	205	118	140	125	41	10.4	14.1	14.0	12.3	40	53	50	39
306	1,500	13.5	6¾	2.58	2.93	.42	.20	28	26	137	229	134	138	185	84	12.0	13.4	9.4	7.3	43	47	42	34
308	1,500	9.7	6	3.52	1.06	.54	.27	43	132	246	278	144	142	124	70	11.6	13.0	14.0	13.8	39	49	48	48
309	1,500	10.3	3	1.58	.90	.60	.43	97	168	250	232	164	158	160	106	12.9	13.5	15.5	18.7	44	47	53	74
311	1,500	11.0	6¾	2.87	2.28	1.98	.19	35	42	48	172	120	116	116	40	10.8	11.2	11.2	14.6	36	38	39	55
265	1,300	9.3	4½	1.53	2.09	.59	.42	94	71	189	102	132	135	103	36	12.7	13.8	15.2	13.8	45		50	38
330	1,250	10.5	6	2.32	1.60	.73	.29	52	85	161	196	134	150	130	64	11.7	12.5	12.8	12.7	40	42	50	47
347	1,250	12.1	7	1.98	1.98	1.49	.30	35	36	63	135	100	110	134	59	13.1	12.2	11.8	15.3	47	48	45	61
Ave. per cent change					+3	−59	−83		+29	+173	+240		+1	−11	−57		+6	+2	+13		+4	+4	+19
P						<.01	<.01			<.01	<.05				<.01								

P.—Is the probability that the observed changes are due to chance (18). Only the statistically significant figures are given. Data on the control series of animals are given in the preceding paper (14). Other details given in footnote, Figure 1.

* Control II was taken 30–60 minutes after control I.

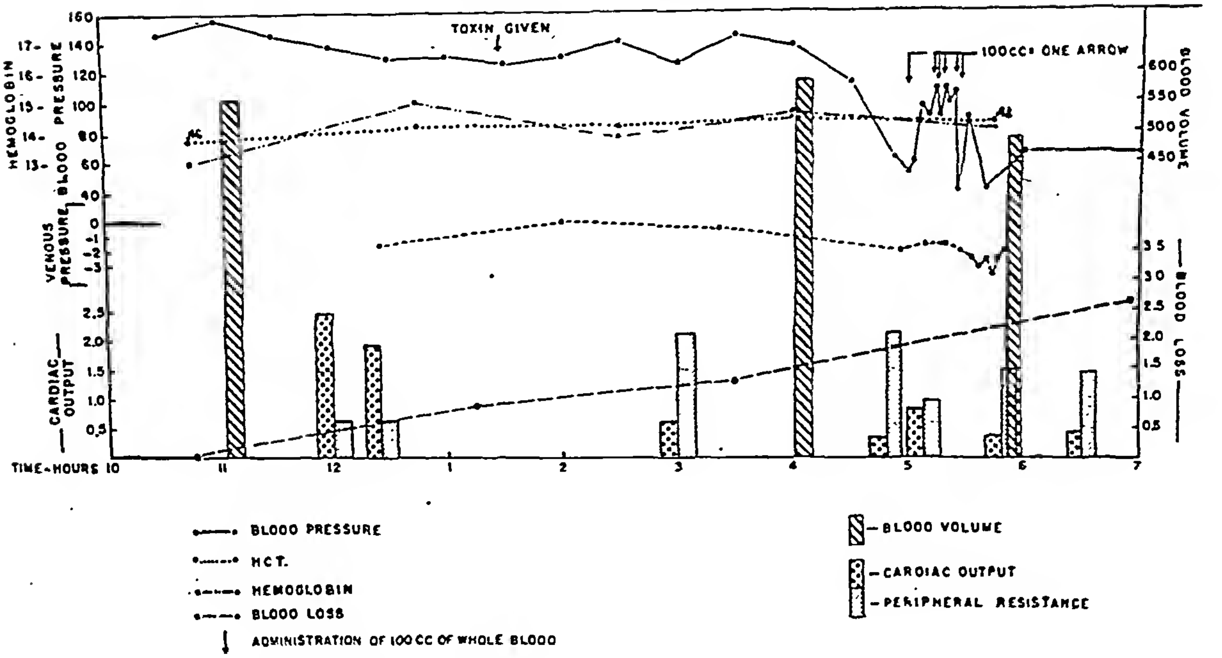

FIG. 1. EFFECTS OF *Cl. oedematiens* TOXIN ADMINISTERED INTRAVENOUSLY

The cardiac output is expressed in liters per minute. The relative peripheral vascular resistance is expressed in arbitrary units as the quotient: blood pressure in mm. mercury/cardiac output in liters per minute. The hemoglobin is expressed as grams per cent. The blood volume is given in cubic centimeters, and the blood loss as per cent of body weight.

ment of a massive edema, which spread from the injection site.

Pathology:[7] The gross autopsy findings associated with intravenous administration of *Cl. oedematiens* toxin in lethal dosage to non-immunized dogs consisted of the following: edema and hemorrhage into the lungs; congestion of the liver; hemorrhage into the adrenals; congestion and submucosal hemorrhage into the small intestine, particularly into the duodenum; small, subendothelial hemorrhages into the cardiac musculature, especially near the attachments of the chordae tendineae. The spleen was uniformly small and contracted. The weights of organs in animals receiving *Cl. oedematiens* and *Cl. welchii* toxins have been recorded in Table II. The liver, lungs, and small intestine showed significant increases in weight, and the spleen a significant decrease, when a lethal dose of *Cl. oedematiens* toxin was given intravenously. If the average change in organ weight is used it appears that there is an increase in weight of the above organs, including the spleen, totalling 2.1 per cent of the body weight. This weight increase, if due to trapping and extravasation of blood into these organs, could represent 20 per cent of the total blood volume.

The microscopic sections revealed a marked engorgement throughout the lungs and viscera, particularly in the former.[8] Capillaries and venules in the submucosal region of the duodenum were extraordinarily dilated and engorged with blood. The mucosa and submucosa of the stomach were spared, and a clear-cut line of demarkation appeared at the pylorus between the relatively normal pallor of the stomach and the red blush of the duodenal mucosa.

Immunized animals (17) injected intravenously with a lethal dose of *Cl. oedematiens* toxin revealed little if any engorgement of blood vessels

[7] According to Doctor A. C. Ivy (15), the capillaries of the duodenum of the dog have been known to be especially sensitive to various toxic substances ingested or produced in the body. The peculiar blood supply of the villi in the duodenal mucosa in the dog (16) appears to offer the best explanation of this phenomenon.

[8] The authors are indebted to Dr. Benjamin Castleman for reviewing and interpreting the microscopic findings.

TABLE II

Mean organ weights, recorded as per cent of body weight

	Liver			Spleen			Small Intestine			Lungs			Heart			Kidneys		
	n	Mean	σ	n	Mean	σ	n	Mean	σ	n	Mean	σ	n	Mean	σ	n	Mean	σ
Controls	11	4.0	.84	11	.55	.15	11	3.0	.38	10	1.05	.13	5	.88	.01	10	.65	.11
Cl. welchii—I.M.‡	17	3.4	.65	17	.44	.19	14	2.9	.66	12	1.08	.48	12	.83	.33	12	.63	.09
Cl. welchii—I.V.‡	7	4.3	1.20	5	.95	.27	3	2.6	.50	5	1.55*	.12	5	.83	.13	5	.79	.19
Cl. oedematiens—I.M.	4	3.5	.28	2	.53	.17	2	3.2	.60	3	1.48	.64	3	.85	.23	3	.56	.15
Cl. oedematiens—I.V.	12	4.9†	.69	11	.37*	.08	10	3.6†	.21	6	1.87*	.64	6	.87	.18	6	.65	.09

I.M. = Intramuscularly.
I.V. = Intravenously.
* = Difference from control value significant ($p < .01$) (20).
† = Difference from control value probably significant ($p < .05$).
‡ = Details of these experiments are given elsewhere (14).
σ = the standard deviation.
n = the number of animals.

in the lungs or viscera. Polymorphonuclear leucocytes were found in considerable numbers in the walls of the pulmonary and adrenal capillaries, presenting a picture similar to that seen in immunized animals injected intravenously with *Cl. welchii* toxin (15).

Cl. oedematiens *toxin intramuscularly* (8 dogs)

Following intramuscular injection of toxin into the thigh muscles in dosages ranging from 150 to 12,000 MLD per kgm., there was a latent period of approximately 6 hours, during which little of significance was observed. From this time on there developed an increasing local edema which spread both up and down the thigh, eventually presenting itself in the retroperitoneal, gluteal, perineal, and neighboring regions. This edema became maximal at 18 to 24 hours, associated with a severe hemoconcentration, the hematocrit reaching values up to 75. The animals became progressively sluggish, developed bloody diarrhea, and died 18 to 38 hours after injection. In 1 dog, injected with a 12,000 MLD per kgm. dose of toxin, death occurred in 10 hours, and the amount of edema was not striking, amounting to 1 per cent of the body weight. This latter finding suggests that death of the animal cannot be accounted for on the basis of local fluid loss alone, but that a generalized toxemia is probably present when the intramuscular dose of toxin is enormous.

In 2 dogs injected with a 150 MLD per kgm. dose of toxin, leg measurements were made, with the following results:

Dog no.	Survival time	Terminal edema (per cent body weight)	Hematocrit	
			Control	Final
388	17 hrs.	5	50	73
324	32 hrs.	7	—	62

The amount of edema was increased if the same quantity of toxin was injected fractionally into several sites.

In order to determine the minimum dosage of toxin which would produce edema by the intramuscular route, all 4 legs were injected in a single dog weighing 13 kgm. The volume of fluid injected into a single limb was kept constant at 5 ml. The dosage of toxin injected ranged, however, from 80 MLD to 10 MLD per kgm. Eighteen hours later, there was massive edema (360 ml.) of the limb where the 80 MLD per kilogram dose had been given; moderate edema (210 ml.) in the limb given 40 MLD per kgm.; minimal edema (70 ml.) in the limb with 20 MLD per kgm.; and no visibly recognizable edema (40 ml. by measurement) in the limb given 10 MLD per kgm.

Pathology associated with intramuscular injection of toxin

Local effects: At the site of injection, there was an area approximately a centimeter in diameter where the muscle substance had been almost completely disintegrated. The surrounding muscle tissue was edematous, with separation of the muscle fibers by edema. Small, flame-shaped hemorrhages in countless number were present along

the course of the dilated and engorged smaller branches of the blood vessels supplying muscle tissue near the injection site. Edema was greatest in the fascial spaces and subcutaneous tissue.

Distant effects: There was 20 to 30 ml. of straw-colored fluid in the pleural and peritoneal cavities. The lungs and visceral organs showed much the same picture although less pronounced than that produced by the intravenous route of injection.

Effect on eyes

One MLD of toxin was injected into the corneae of each of 4 rabbits' eyes.[9] Eighteen hours after injection, there was marked chemosis of the conjunctival blood vessels. By means of slit lamp illumination and the high-power dissecting microscope, tiny flame-shaped hemorrhages were seen extending out from a number of the vessels. There were dilatation and engorgement of all vessels.

DISCUSSION

Experiments on the effect of *Cl. welchii* toxic filtrates (14) have demonstrated that the dosage, rate, and route of administration of the toxins determine the type of effects on dogs. The same considerations apply to the present studies on *Cl. oedematiens* toxin. Rapid intravenous administration of a large dose of toxin is a situation far different from the slow elaboration of the toxin by organisms multiplying in a damaged muscle. Intramuscular injection of a small, sub-lethal dose of toxin appears to approach the clinical situation most closely. Here the effect of the toxin is principally local, resulting in edema and hemoconcentration. Such a picture of *Cl. oedematiens* myositis, predisposing to wound shock, has been observed in forward field hospitals (18).

The data gathered on the effects of this toxin after intravenous injection, are consistent with the interpretation that the principal site of its action is on the peripheral vascular system. The toxin appears to act by causing loss of tone and damage to capillaries and other small blood vessels. As a result, the smaller vessels of the lungs and viscera become engorged with blood which then extravasates into surrounding tissues. The quantity of blood actively circulating is thereby decreased, and a fall in cardiac output occurs. There is a compensatory peripheral vasoconstriction, probably in the arterioles, resulting in an increased peripheral resistance. The extravasation of fluid and blood into the pulmonary alveoli produces an interference with oxygenation of the blood which eventually assumes critical proportions. The blood pressure remains unchanged until a point is reached where trapping of blood out of the active circulation and interference with oxygenation become too great for compensation. The blood pressure then falls to shock levels and death follows.

The possibility that the effects observed may be a non-specific foreign protein reaction is ruled out by the protective effect of antitoxin when given intravenously simultaneously with the toxin and by the complete protection against either intravenously or intramuscularly injected toxin provided by active immunization (17).

When the toxin is injected intravenously, blood vessels throughout the animal are exposed to the toxin, and the lungs and visceral organs suffer particularly. On intramuscular injection, however, blood vessels adjacent to the injection site are exposed first and come into contact with the greatest concentration of toxin. This correlates with the observation that edema into the injected area becomes the outstanding feature.

The clinical picture which this toxin produces when injected intravenously is that of shock—apparently as a consequence of 2 factors: (*1*) trapping of whole blood in the viscera, rendering it unavailable to the circulation as a whole; and (*2*) extravasation of plasma and whole blood into vital organs through damaged capillaries. Addition of fluid to the circulatory system under these conditions does not restore the animal to normal, although it has a temporary beneficial effect until the additional fluid has likewise been lost from the circulation.

The inability of antitoxin to protect the animal when given more than a few minutes after the toxin suggests that the toxin produces some irreversible action on the blood vessel wall. One is tempted to reason from analogy with the action of *Cl. welchii* alpha toxin that *Cl. oedematiens* toxin (which appears to be protein in nature) may be an enzyme which acts on cell surfaces, destroy-

[9] These experiments were carried out in collaboration with Dr. David Cogan.

ing some architectural unit of the surface structure. Once the damage is done, antitoxin may neutralize the effects of the toxin still circulating, but the initial damage is irreparable.

The possibility that myocardial failure might contribute to the circulatory failure has not been completely eliminated in these experiments. However, it has been shown by means of heart-lung preparations (19) that the cardiac ability and reserve are adequate up to the very end.

SUMMARY

Cl. oedematiens toxin administered intravenously produced the following picture: a latent period of one or more hours without noticeable change, followed by a progressive rise in peripheral vascular resistance, a fall in cardiac output, and eventually a decline in blood pressure to shock levels and death. Edema and hemorrhage in the lungs and visceral organs were constant findings. *Cl. oedematiens* toxin produced massive local edema, hemoconcentration, and eventual death, when administered intramuscularly in small dosage.

Intravenous fluids were of no permanent benefit in this syndrome. Under the conditions used, antitoxin did not prevent the lethal outcome if given more than 5 minutes after injection of the toxin.

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BIBLIOGRAPHY

1. MacLennan, J. D., Anaerobic infections of war wounds in the Middle East. Lancet, 1943, 2, 63, 94, 123.
2. Novy, F. G., Ein neuer anaërober Bacillus des malignen Oedems. Ztschr. f. Hyg. u. Infectionskrankh, 1894, 17, 209.
3. Weinberg, M., and Seguin, P., Le B. oedematiens et la gangrène gazeuse. Compt. Rend. Soc. de Biol., 1915, 78, 507.
4. Sacquépée, M. E., Études sur la gangrène gazeuse, Le bacille de l'oedème gazeux malin. Ann. Inst. Pasteur., 1916, 30, 76.
5. Guillaumie, M., Inhibition de l'effet protéolytique de la toxine oedematiens. Compt. rend. Soc. de Biol., 1942, 136, 479.
6. Favata, B. V., Dowdy, A. H., Sewell, R. L., and Vincent, J. G., The pathology of experimental clostridial infections in dogs. Surg., Gynec., Obst., 1944, 79, 660.
7. Aub, J. C., Brues, A. F., Dubos, R., Kety, S. S., Nathanson, I. T., Pope, A., and Zamecnik, P. C., Bacteria and the "toxic factor" in shock. War Med., 1944, 5, 71.
8. Nathanson, I. T., Nutt, A. L., Pope, A., Zamecnik, P. C., Aub, J. C., Brues, A. M., and Kety, S. S., The toxic factors in experimental traumatic shock. I. Physiologic effects of muscle ligation in the dog. J. Clin. Invest., 1945, 24, 829.
9. Brues, A. M., Cohn, W. E., Kety, S. S., Nathanson, I. T., Nutt, A. L., Tibbetts, D. M., Zamecnik, P. C., and Aub, J. C., The toxic factors in experimental traumatic shock. II. Studies on electrolyte and water balance in shock. J. Clin. Invest., 1945, 24, 835.
10. Kety, S. S., Nathanson, I. T., Nutt, A. L., Pope, A., Zamecnik, P. C., Aub, J. C., and Brues, A. M., The toxic factors in experimental traumatic shock. III. Shock accompanying muscle ischemia and loss of vascular fluid. J. Clin. Invest., 1945, 24, 839.
11. Aub, J. C., Brues, A. M., Kety, S. S., Nathanson, I. T., Nutt, A. L., Pope, A., and Zamecnik, P. C., The toxic factors in experimental traumatic shock. IV. The effects of the intravenous injection of the effusion from ischemic muscle. J. Clin. Invest., 1945, 24, 845.
12. Zamecnik, P. C., Aub, J. C., Brues, A. M., Kety, S. S., Nathanson, I. T., Nutt, A. L., and Pope, A., The toxic factors in experimental traumatic shock. V. Chemical and enzymatic properties of muscle exudate. J. Clin. Invest., 1945, 24, 850.
13. Pope, A., Zamecnik, P. C., Aub, J. C., Brues, A. M., Dubos, R. J., Nathanson, I. T., and Nutt, A. L., The toxic factors in experimental traumatic shock. VI. The toxic influence of the bacterial flora, particularly *Clostridium welchii*, in exudates of ischemic muscle. J. Clin. Invest., 1945, 24, 856.
14. Zamecnik, P. C., Nathanson, I. T., and Aub, J. C., Physiologic action of *Clostridium welchii* (Type A) toxins in dogs. J. Clin. Invest., 1947, 26, 394.
15. Ivy, A. C., Personal communication.
16. Klemperer, P., Penner, A., and Bernheim, A. I., The gastro-intestinal manifestations of shock. Am. J. Digest. Dis., 1940, 7, 410.
17. Nathanson, I. T., Levin, J., Aub, J. C., and Zamecnik, P. C., Unpublished data.
18. Stewart, J. D., and Warner, F., Observations on the severely wounded in forward field hospitals with special reference to wound shock. Ann. Surg., 1945, 122, 129.
19. Krayer, O., Aub, J. C., Nathanson, I. T., and Zamecnik, P. C., The influence of antitoxin upon the action of *Clostridium oedematiens* toxin in the heart-lung preparation of the dog. J. Clin. Invest., 1947, 26, 411.
20. Arkin, H., and Colton, R. R., An Outline of Statistical Methods, 4th edition. De Pamphilis Press, Inc., New York, 1939, p. 127.

THE INFLUENCE OF ANTITOXIN UPON THE ACTION OF *CLOSTRIDIUM OEDEMATIENS* TOXIN IN THE HEART-LUNG PREPARATION OF THE DOG [1, 2, 3]

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Previous experiments (1) have indicated that *Cl. oedematiens* toxin produces types of dynamic effects similar to those seen in traumatic shock. Blood volume was not diminished to the same extent as in hemorrhagic shock, but the peripheral vascular resistance rose and the cardiac output was reduced in the characteristic manner. It was not obvious, in the intact dog, whether these difficulties arose in the peripheral circulation or in the heart. Wiggers (2) has discussed a similar type of problem with reference to shock. Thus, in order to evaluate more specifically the factors involved in the circulatory failure resulting from *Cl. oedematiens* toxin, the heart-lung preparation has been utilized.

METHODS

The experiments were carried out on the isolated heart and lungs of the dog in the form of the heart-lung preparation as described by Patterson and Starling (3). Mongrel dogs were used in all experiments and weighed between 7.8 and 11.3 kgm. They were anesthetized for the surgical procedure with sodium pentobarbital in a dose of 35 mgm. per kgm. body weight. The period of preparation was timed so as to be about of equal duration in all experiments and was approximately 1 hour from the time of the first skin incision to the start of recording. The blood temperature varied in the various experiments not more than between 37 and 39° C. and in the same experiment not more than ± 1° C. The method of recording right auricular pressure, pulmonary pressure, and output is described in detail by Krayer and Mendez (4). In the various experiments arterial resistance was between 75 and 85 mm. Hg, assuring a mean aortic pressure of about 100 mm. Hg when the systemic output under basal conditions of blood supply was kept between 400 and 500 ml. (systemic output is output of left ventricle minus coronary flow). The competence of the heart was tested by increasing venous blood supply. This was achieved by raising the level of the blood in the venous reservoir in two steps of 50 mm. each as described in detail by Krayer (5). In the normal heart, at the beginning of the experiment, this led to an increase of systemic output of 40 to 50 per cent of the basal value at the first step, and of 80 to 95 per cent of the basal value at the second step. While the main increase in the work of the heart thus represented an increase in volume work, a certain amount of increase in pressure work was also involved in this competence test, as the arterial resistance was not changed throughout the experiment; and the mean blood pressure, therefore, increased somewhat with an increase in systemic output; likewise pulmonary arterial pressure increased. The increase of pulmonary pressure in experiments of this type becomes more and more pronounced as anatomical changes begin to appear and eventually lead to lung edema. In the present series of experiments, competence tests were made at intervals of approximately 30 minutes until the limit of competence was reached. By definition we consider this to be the case when an increase in venous supply by raising the level of blood in the venous reservoir by 50 mm. no longer leads to an increase in systemic output (5).

Defibrinated blood, taken from a bleeder dog, was used to fill the heart-lung system. The total blood volume usually amounted to 800 to 900 ml. at the beginning of the experiments.

Hematocrit determinations were done by taking 10 ml. of defibrinated blood from the heart-lung system and subjecting the blood to centrifugation at 2,400 revolutions per minute for 15 minutes.

Artificial respiration was carried out with the Starling respiratory pump, using room air; the stroke volume was kept between 125 and 150 ml., and the rate of ventilation was between 14 and 18 per minute.

Twenty-one experiments were conducted, in 16 of which *Cl. oedematiens* toxin was administered. This was given into the blood stream before it entered the venous reservoir, to ensure good mixing with the blood before it

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[1] This is publication No. 632 of the Cancer Commission of Harvard University, and No. X in the series entitled "The Toxic Factors in Experimental Traumatic Shock."

[2] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital.

[3] Grateful acknowledgment is due the Josiah Macy, Jr. Foundation for a grant for the histopathological studies.

reached the heart. The dose was 10 or 20 mgm. of *Cl. oedematiens* toxin preparation (45N), 2,500 mouse subcutaneous MLD per mgm., or 100 mgm. *Cl. oedematiens* toxin (N313), 500 mouse subcutaneous MLD per mgm.[4] The material was dissolved prior to the administration in 10 ml. of 0.9 per cent sodium chloride solution and/or in phosphate buffer of pH 7.4. The administration of the toxin was made at the basal inflow level and within a period of about 1 minute. Histamine content of the 2 toxin samples was checked with the use of isolated strips of guinea pig's ileum. The highest concentration tested was 1:15,000. The rise in tone of the gut was not characteristic and was very much less than that due to histamine dihydrochloride 1:60 million.

The antitoxin was a polyvalent preparation (Lederle). It was given in a dose of 7 ml. in 8 experiments and was administered within approximately 30 seconds.

Dogs in 3 experiments were actively immunized against *Cl. oedematiens* toxin according to technics described in detail elsewhere (6). In one of the experiments (Experiment No. 7) the bleeder dog as well as the dog used for the heart-lung preparation was actively immunized, and no antitoxin administered during the course of the experiment. The immunization was carried out as follows: the bleeder dog was immunized with four 1-ml. subcutaneous injections of *Cl. oedematiens* toxoid (Lederle No. 100 × 18A), injections being given at 3-week intervals. The heart-lung preparation dog (H.L.P.) was immunized similarly, with the exception that injections were given at weekly intervals. In Experiment No. 16 the H.L.P. dog was immunized. The bulk of the immunized blood was removed, 500 ml. of blood from a normal dog was then given into the H.L.P. and again removed to the greatest extent ("washout"), and fresh blood from a normal dog was then added to carry out the experiment. In Experiment 17, the H.L.P. dog was a normal animal. The normal blood was removed and the "immunized" blood from the H.L.P. dog of Experiment 16, together with the "washout," was used to carry out the experiment. The H.L.P. dog of Experiment 16 was immunized with *Cl. oedematiens* toxoid (Novyi, No. 101 × 18A, Lederle Laboratories). The dog was given 9 subcutaneous injections of 1 ml. of this toxoid at weekly intervals. In addition it received a dose of 1,250 MLD *Cl. oedematiens* toxin per kgm. intramuscularly, as a booster dose, a week before it was used for the heart-lung preparation.

Five experiments within this series were carried out as controls, using the identical procedure of preparation and testing of competence, but without administering either antitoxin or toxin.

At the end of several experiments, specimens of heart and of lung tissue were taken for pathological studies. They were fixed in Zenker's fluid and were stained with phloxine-methylene blue.

[4] The authors are indebted to Professor Milan A. Logan, University of Cincinnati, for generous supplies of *Cl.*

RESULTS

1. *The action of toxin*

(All the results are given in Figure 1.) When toxin was added to the blood of the heart-lung preparation, no noticeable change in right auricular, pulmonary pressure, or competence occurred for a period of 30 to 50 minutes. After this, within a period of a quarter of an hour, pulmonary pressure increased and simultaneously the lungs became more rigid, indicating the onset of lung edema. In 3 experiments (Experiments 1, 2, and 3) with a dose of 10, 20, and 20 mgm. of 45N toxin respectively, severe lung edema terminated the experiment within 65 to 75 minutes after the addition of the toxin. In Experiments 18 and 19, after the addition of 100 mgm. of *Cl. oedematiens* toxin N313, severe lung edema terminated the experiment after 51 and 52 minutes respectively. No test of competence was performed in Experiment 19 after the administration of the toxin. As can be seen from Figure 1, heart-lung preparations made in the same way but not under the influence of toxin have a long survival time. Five control experiments (Nos. 13, 14, 15, 20, and 21) are plotted in the graph, so that the time 30 minutes after starting the recording arbitrarily was called zero time (since the time between the start of recording and addition of toxin in all the toxin experiments in Figure 1 averaged approximately 30 minutes). In 4 control experiments (13, 14, 15, and 20) it took between 140 and 165 minutes to reach the limit of competence; in Experiment 21, it took 210 minutes. The limit of competence was used as an end point, since, as a rule, lung edema does not terminate a heart-lung preparation before the end of 3 hours or more. Signs of marked lung edema were present only in Experiment 15.

2. *The influence of antitoxin upon the toxin action*

In the 3 experiments with 10 mgm. of toxin 45N and antitoxin (Experiments 4, 5, and 6 of Figure 1), the antitoxin was administered at varying intervals: 73 and 10 minutes before, and 17 minutes after the toxin. In all 3 cases the limit of competence was reached within 142 to 160 minutes, a period of survival significantly longer than in the experiments without antitoxin. Signs of lung edema were marked in Experiment 4. In Experiment 12, in which antitoxin was given 10 minutes

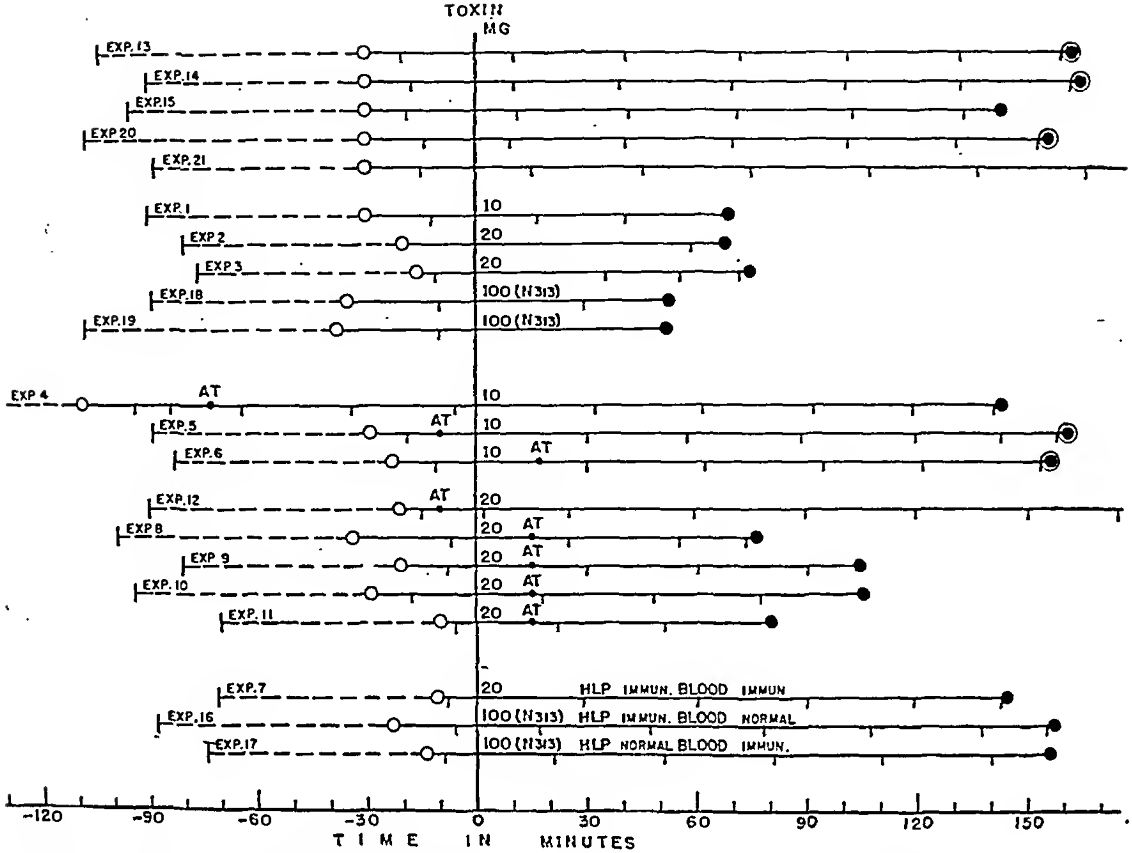

FIG. 1. THE ACTION OF *Clostridium oedematiens* TOXIN UPON THE HEART-LUNG PREPARATION OF THE DOG

The dotted line from the vertical bar to the open circle indicates the time of preparation from skin incision to start of recording. The large full circle at the end of every horizontal line indicates lung edema. Limit of competence reached without marked signs of lung edema is indicated by the full circle placed in an open circle. The small full circle at the letters AT indicates administration of antitoxin. The figures 10, 20, and 100 respectively at the vertical zero time line indicate the administration of 10 or 20 mgm. of toxin (45N) or 100 mgm. of toxin (N313). The signals below every horizontal line indicate the beginning of a competence test. For further information, see text.

prior to the dose of 20 mgm. toxin 45N, the limit of competence was reached only after 200 minutes. On the other hand, in the 4 experiments, 8, 9, 10, and 11, in which antitoxin was given 15 minutes after 20 mgm. of toxin 45N, severe lung edema occurred within 76, 80, 104, and 105 minutes respectively.

3. *Pathological changes*

The microscopic sections of the lungs, in preparation injected with toxin alone, revealed the presence of engorged pulmonary capillaries, and red cells and protein within the pulmonary alveoli. Where antitoxin was given prior to the toxin, the lungs at termination of the experiment (except in Experiment 4) showed either an absence of these findings or their presence to a minor degree. In all cases, the heart on microscopic examination showed no characteristic pathological changes.

4. *The influence of active immunization*

An attempt was made to obtain information on the effect of active immunization upon the response of the heart-lung preparation to toxin (Experiments 7, 16, 17 [Figure 1]). Although 2 different samples of toxin were used, the potency was identical in all 3 experiments (50,000 MLD mouse units). A definite inhibiting effect of immunization to the toxin action was observed not only when the blood as well as the heart and lungs were taken from immunized dogs (Experiment 7) but also when either the H.L.P. (Experi-

ment 16) or the blood (Experiment 17) was from an actively immunized dog.

The alterations of the hematocrit in the heart-lung preparation with respect to administration of *Cl. oedematiens* toxin are tabulated in Table I. The available data suggest that the hematocrit is not a good index of the onset of the pulmonary changes, probably because whole blood as well as plasma is lost into the pulmonary alveoli.

TABLE I

Relationship of hematocrit to administration of Cl. oedematiens *toxin, in the heart-lung preparation*

Dog no.	Dose of toxin in mouse MLD	Time antitoxin given	Hematocrit	
			Original	Terminal
4	25,000	72 min. before toxin	50	41
5	25,000	10 min. before toxin	50	58
6	25,000	17 min. after toxin	61	68
7*	50,000	none given	44	47
16*	50,000	none given	54	61
17**	50,000	none given	64	70
8	50,000	15 min. after toxin	65	69
9	50,000	15 min. after toxin	59	59
18	50,000	none given	46	74
19	50,000	none given	48	61

* Immunized dog.
** Blood from immunized dog used.

DISCUSSION

Under the conditions of our experiments, the action of *Cl. oedematiens* toxin is essentially on the pulmonary vessels, producing changes which lead to loss of blood into the lungs. While it cannot be said that the heart muscle is not affected, changes in work of the left heart by increasing the arterial resistance indicate that the left ventricle was relatively little affected. It was still capable of additional work at a time when an increase in venous blood supply led to a marked rise in pulmonary pressure so that lung edema was hastened and it became difficult or impossible to increase venous inflow.

The effect of the toxin does not occur quickly; rather, a latent period of considerable duration elapses before definite changes in right atrial pressure, in pulmonary pressure, or in competence become noticeable. This places these toxin effects in a group of delayed reactions characteristic of various types of other pharmacological agents, for instance, the cardiac glycosides and the thyroid hormone. One has to assume, in these instances, that the substance itself has to enter into or initiate a chain of reactions requiring considerable time before the characteristic effect eventually occurs. Since the toxin appears to be protein in nature, the question may be raised as to whether it may not be an enzyme. The identification of the *Cl. welchii* alpha toxin as a lecithinase (7) provides the best example of a bacterial toxin which is enzymic in nature.

The experiments appear to indicate that 7 ml. of antitoxin was able to afford a considerable degree of protection against the toxic effect of 25,000 MLD of toxin irrespective of whether the antitoxin was administered prior to the toxin or 17 minutes after the toxin. Protective action was also present in the experiments in which injection of antitoxin preceded the administration of 50,000 MLD of toxin. Of the 4 experiments in which the antitoxin followed 15 minutes after the dose of 50,000 MLD of toxin, no protection could be seen in 2; severe lung edema developed in approximately the same period of time as in the experiments without antitoxin. In the 2 other experiments it is possible that some delay in the onset of pulmonary changes may have been caused by the antitoxin.

Protection against the action of 50,000 MLD of toxin was observed in the 3 experiments in which the heart and lungs or the blood used was obtained from immunized animals. While it is not possible to say that all immunized blood was removed from the H.L.P. of Experiment 16, the amount of blood-immune bodies must have been greatly reduced by replacing the blood twice with blood from a non-immunized animal. However, this experiment is not a definite proof that tissue-immune bodies are sufficient to counteract the toxin action even if the blood is not immunized. On the other hand, immunized blood alone appears to give protection (Experiment 17).

SUMMARY

1. In the heart-lung preparation of the dog, *Cl. oedematiens* toxin produces pronounced loss of plasma and whole blood in the pulmonary circulation. This phenomenon is observed only after a definite latent period.

2. The effects on the heart are minimal in comparison, for the heart is capable of increased work

at the time when the pulmonary circulation is greatly impaired.

3. Antitoxin counteracts the change in permeability of the pulmonary vessels if it is present in the system when toxin is given. It is less effective when given early in the latent period.

4. Active immunization markedly diminishes the toxic effects on the pulmonary circulation. In the immunized dog the antitoxin is definitely present in the circulating blood, and the evidence is suggestive of the supposition that it may be present in the heart and lung tissue.

BIBLIOGRAPHY

1. Aub, J. C., Zamecnik, P. C., and Nathanson, I. T., Physiologic action of *Clostridium oedematiens* (Novyi) toxin in dogs. J. Clin. Invest., 1947, 26, 404.
2. Wiggers, C. J., The present status of the shock problem. Physiol. Rev., 1942, 22, 74.
3. Patterson, S. W., and Starling E. H., On the mechanical factors which determine the output of the ventricles. J. Physiol., 1914, 48, 357.
4. Krayer, O., and Mendez, R., Studies on veratrum alkaloids. I. The action of veratrine upon the isolated mammalian heart. J. Pharmacol. and Exper. Therap., 1942, 74, 350.
5. Krayer, O., Versuche am insuffizienten Herzen. Arch. f. exper. Path. u. Pharmakol., 1931, 162, 1.
6. Nathanson, I. T., Rubin, J., Aub, J. C., and Zamecnik, P. C., Unpublished data.
7. Macfarlane, M. G., and Knight, B. C. J. G., The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. Biochem. J., 1941, 35, 884.

THE SCATTER AND ABSORPTION OF LIGHT BY THE EXCISED CORNEA AT VARIOUS WAVELENGTHS DETERMINED BY A PHOTOELECTRIC METHOD. THE EFFECT OF TRAUMA AND VACCINIAL INFECTION

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The effects of immunity and x-ray therapy on the extent of permanent damage to the cornea resulting from the invasion of the cornea by vaccinia have not previously been subjected to experimental study. In preliminary experiments (1) an attempt was made to judge the effects of such treatment by grading the opacities according to an arbitrary scale. It was soon realized that this method was inadequate. In order to obtain a more accurate evaluation of therapy, it was necessary to devise a procedure for estimating objectively the degree of corneal opacity. By utilizing a technique which measures the per cent transmission of light by the cornea *in vitro*, it was possible to estimate the degree of impairment to vision resulting from diminished light transmission due to opacities. This procedure is described here, and in addition data are given on the light transmission of normal rabbit corneas, corneas subjected to trauma, and corneas inoculated with vaccinia but not treated. The effects of therapy will be described elsewhere (1).

PROCEDURE

The degree of opacity was estimated by measuring the transmission of light through the cornea in the Evelyn photo-electric micro-colorimeter (2). The intensity of the incident beam of light was adjusted so that the galvanometer read 100 per cent transmission when the light was passed through 0.9 per cent NaCl solution. Within 15 minutes after the rabbit was sacrificed the cornea was placed in the cell with the convex surface downward. The plunger was inserted, and the per cent transmission of the light was read. The plunger was adjusted to rest lightly on the cornea thus causing it to lie flat. Appropriate filters (red, green and blue[1]) were used so that the visual spectrum was covered at the intervals given in Tables I and II. At least two determinations of transmission were always made on each cornea at each wavelength.

It usually was not necessary to trim the cornea to fit the cell, but on occasion this was necessary. The trimmed edge always showed some opacity resulting from the trauma, but care was taken to exclude the edge from the path of the light beam. After insertion of the plunger the cornea was inspected to exclude the presence of air bubbles and foreign material.

In only a few instances did the opacity cover the entire field. In most instances the opacity was relatively small in comparison with the field, and in these cases the opacity was centered in the field.

Experiments reported here include studies made upon normal rabbit corneas (group Q); corneas which had been anesthetized with a 2 per cent butyn sulfate solution, scratched with a needle and treated with 105 r of x-ray (group L); untraumatized corneas removed from rabbits in which the other eye had been inoculated with vaccinia (group P); and corneas which had been inoculated with vaccinia, but in which no form of therapy was given (group K). Beginning 25 days after the start of the experiment one animal from each group was sacrificed on successive days and the measurements were made. Preliminary experiments (1) had shown that this lapse of time was sufficient for the development of permanent opacities.

RESULTS

Table I shows that in the normal corneas there was a progressive decrease in the average transmission of light as the filters were changed to allow the transition from long to short wavelengths. This decrease could be due to the absorption of light by the cornea, since Wald (3) states that there is some absorption by the normal cornea in the violet region of the spectrum. It could also be due to the scattering of light by the cornea. Rayleigh's law states that the intensity of the scattered light is inversely proportional to the fourth power of the wavelength; therefore, the scattered radiation would be greatly increased at the shorter wavelengths. The data given in Table I have

[1] The filters used were obtained from the manufacturer the microcolorimeter and were designated by the wave-[illegible] in millimicrons of the maximum light transmitted. [illegible]he limits of transmission are indicated in Table I.

TABLE I

The per cent transmission of light (T) by normal rabbit corneas, Group Q, at different wavelengths

No.	*F660 mμ †(635–720 mμ)	F620 mμ (595–660)	F540 mμ (515–570)	F520 mμ (495–550)	F490 mμ (465–530)	F440 mμ (410–475)	F420 mμ (380–460)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	99.50	99.50	98.00	97.50	97.50	95.00	93.00
2	99.00	99.00	97.25	97.00	96.00	93.50	91.50
3	99.00	98.75	97.00	96.00	96.00	93.50	92.00
4	97.00	96.00	94.25	93.75	92.00	90.00	88.00
5	99.00		97.50	96.50			92.00
6	99.25		97.25				
7	99.25		97.25	97.00	96.00		92.00
8	99.25		96.50	93.00	92.50	89.00	88.00
9	98.75		97.00	96.50	96.00	93.50	92.00
10	98.50		96.50	96.00	95.50	93.00	91.00
Mean	98.9	98.1	96.9	95.4	95.2	92.5	91.0

* Wavelength of maximum transmission.
† Wavelength transmission limits.

been examined from this viewpoint to see if scattering is a factor.

If T represents the per cent of light transmitted by the cornea, then $100 - T$ would represent the per cent scattered. According to Rayleigh's law then

$$(100 - T) = k/\lambda^4.$$

The constant k was calculated for $1/\lambda^3$, $1/\lambda^4$, and $1/\lambda^5$, using the observed data for the value of $100 - T$ at $\lambda = 420$ mμ, and the above formula. With these values for k, theoretical lines were constructed to show the anticipated relationship between $100 - T$ and the above reciprocal functions. Figure 1 shows that when the observed values for $100 - T$ were plotted against $1/\lambda^4$ for each wavelength the observed data fell either on or very close to the theoretical line. Using $1/\lambda^3$ the observed values were consistently considerably less than those expected from the theoretical line, and using

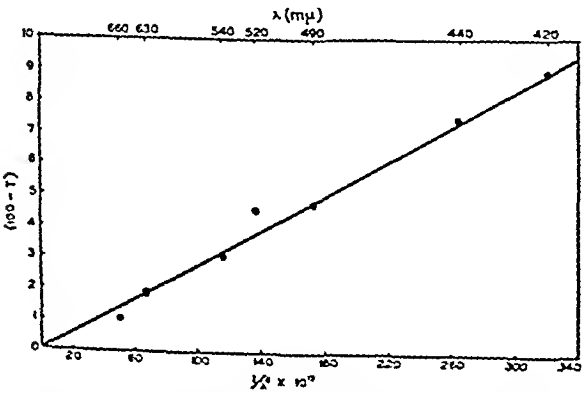

FIG. 1. RELATIONSHIP BETWEEN THE OBSERVED VALUES OF $(100 - T)$, OBTAINED ON NORMAL CORNEAS (GROUP Q), AND THE THEORETICAL LINE CONSTRUCTED FROM THE EQUATION $(100 - T) = k/\lambda^4$. WHEN k, CALCULATED FROM THE OBSERVED VALUE OF $(100 - T)$ AT $\lambda = 420$ mμ, WAS 28×10^{10}

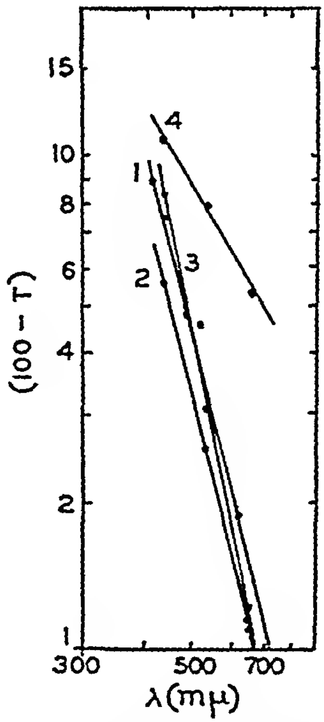

FIG. 2. RELATIONSHIP BETWEEN AVERAGE OBSERVED VALUES OF $(100 - T)$ AND THE WAVELENGTH OF LIGHT
(1) normal corneas, slope = − 4.0
(2) Group P, slope = − 4.0
(3) Group L, slope = − 4.7
(4) Group K, slope = − 1.8

$1/\lambda^4$ they were consistently considerably greater than the expected values at all wavelenths greater than 420 mμ. This is illustrated further by the fact that when the observed values for $100 - T$ were plotted against the wavelengths on double logarithmic paper (Figure 2) the slope of the resulting line was minus 4, indicating that $100 - T$ is proportional to $1/\lambda^4$. Thus it appears that the data obtained on normal rabbit corneas obey Rayleigh's law, and that the decrease in transmission at the shorter wavelengths is due to scattering of light rather than to absorption.

The data obtained for the transmission of light by the untraumatized corneas (group P), the corneas which were anesthetized, scratched and treated with x-ray (group L), and the corneas which were inoculated with vaccinia but were untreated (group K) are presented in Table II. When the average

TABLE II

The per cent loss of transmission of light by rabbit corneas

Group K: inoculated with vaccinia, untreated; group L: anesthetized, scratched with needle and subjected to x-ray; group P: untraumatized corneas from opposite eye of rabbits with ocular vaccinia

Group	100-T F660			100-T F540			100-T F440		
	K	L	P	K	L	P	K	L	P
1	5.50	1.75	1.25	10.25	4.00	2.50	46.00		5.50
2	2.75	1.25	1.75	4.00	3.00	2.75	18.00	6.50	5.50
3	3.75	0.75	1.00	6.00	2.50	2.75	8.00		5.50
4	23.75	1.75	2.50	32.00	3.50	3.50	6.00		5.00
5	7.25	1.00	0.75	11.00	2.50	1.75	11.50		6.00
6	0.50	1.50	0.75	3.75	3.75	2.50	10.50		5.50
7	1.75	1.50	1.00	3.25	3.50	2.50	14.50		5.50
8	3.50	0.75	1.00	6.50	3.50	3.00	8.00	7.00	7.00
9	4.25	1.25	0.00	6.50	2.50	2.00	10.00	11.25	4.50
10	5.00	0.75	0.50	8.00	2.75	3.00	12.00		5.00
11	3.75		0.75	4.50		3.00	9.00		6.50
12	4.25		1.25	6.00		3.00	6.00		6.00
13	5.50		0.50	7.50		2.50			
14	3.50		0.25	5.25		2.00			
15	1.00		2.00	2.25		3.00			
16			0.00			1.75			
17			2.00			2.50			
18			1.50			2.75			
19			2.75			3.50			
Mean	5.06	1.23	1.13	7.78	3.15	2.64	13.29		5.63
N	15	10	19	15	10	19	12		12
σ	5.46	0.40	0.79	7.13	0.57	0.51	10.86		0.68

values for $100 - T$ in these groups were plotted against the wavelengths, using double logarithmic aper (Figure 2), it was found that the lines for roups P and L did not deviate significantly from slope of minus 4. The slight deviation for group is within the limits of experimental error. The lope for group K (inoculated untreated corneas) owever has a value of minus 1.8, which indicates hat some factor in addition to the scatter diminishes the transmission of light when there is an opacity present. In order to get further information about this unknown factor the values obtained on a number of animals in group *K* were plotted on double logarithmic paper. Figure 3 shows that the slope varied from minus 4.5 for the least degree of opacity to minus 1.6 for the greatest degree of opacity. This variability in slope is primarily due to a greater decrease in transmission at the longer wavelengths with increasing opacity.

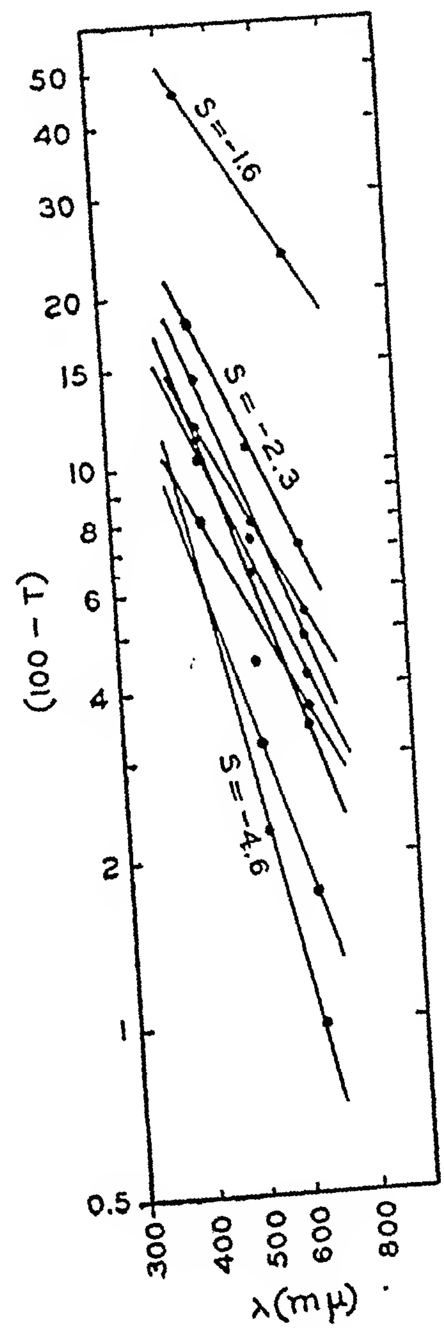

FIG. 3. RELATIONSHIP BETWEEN $(100 - T)$ AND THE WAVELENGTH, FOR 9 OF THE CORNEAS FROM GROUP K. $s =$ SLOPE

The significance of the difference between the mean values of $100 - T$ (Table II) for groups L and P was tested by "Student's" t-test (4) and was found to be not significant for filter 660 (red). For filter 540 (green), the difference was significant at the 5 per cent level only. The difference between the means for groups P and K was significant at the 1 per cent level for filters 660, 540, and 440 (blue).

DISCUSSION

The procedure described here is a simple objective means for determining the effect of a corneal opacity on the transmission of light at different wavelengths by the cornea. In a few instances no opacity was noticed upon visual inspection of the cornea *in situ;* however, the transmission of light was impaired in these corneas, and upon careful inspection a rather diffuse opacity was seen.

It is apparent from these experiments that the decrease in transmission of light by the normal cornea at the shorter wavelengths is probably due to a scattering of the incident light by the cornea. This finding raises the question as to whether or not there is any absorption of light by the normal cornea, as reported by Wald (3). It is also apparent that the presence of an opacity introduces some factor, in addition to scatter, which results in a greater defect of transmission. The nature of this factor is unknown, but the data presented in Figure 3 could be explained by the addition of a neutral filter which would absorb equally at all the wavelengths. If this were true the relative effect of the filter would be greatest at the long wavelengths, because at these wavelengths the effect of scatter is minimal. We would therefore suggest that the effect of an opacity on transmission is due to the equal absorption of light at all wavelengths by the opacity.

The comparison of the means for groups L and P indicates that the trauma associated with anesthesia, scratch, and x-ray produces some permanent decrease in the transmission of light by the cornea, even in the absence of evidence of an opacity. The lack of significance in the difference of the means for filter 660 is probably due to the minimal effect of scattering at the long wavelengths together with the factor of sampling variation. It is clear from Figure 2 that trauma without resulting opacity does not alter the nature of the transmission defect found in normal corneas, since the data for group L obey Rayleigh's law.

SUMMARY

1. The *in vitro* transmission of light by the rabbit cornea was measured with the Evelyn photoelectric microcolorimeter.

2. In the normal cornea the per cent transmission is less at the short than at the long wavelengths of light. Since the decrease in transmission is proportional to $1/\lambda^4$ and is consistent with Rayleigh's law, it is concluded that the decrease is due to scattering of the incident light.

3. Trauma to the eye may result in a permanent impairment of light transmission in the absence of an opacity which can be seen by inspection. This decrease is also due probably to scattering of light rather than to absorption.

4. Inoculation of the rabbit cornea with vaccinia results in opacity which causes a significant decrease in the transmission of light. This decrease is probably due to equal absorption of light at all wavelengths.

BIBLIOGRAPHY

1. Harrell, G. T., Reid, C. H., Little, J. M., Mankin, J. W., Pittman, H. W., Holt, L. B., and Morris, L. M., The effect of roentgen therapy on experimental ocular vaccinia in non-immune and partially immune rabbits. Arch. Ophth. In press.
2. Evelyn, K. A., and Cipriani, A. J., Photoelectric microcolorimeter. J. Biol. Chem., 1937, 117, 365.
3. Wald, G., Human vision and spectrum. Science, 1945, 101, 653.
4. Snedecor, C. W., Statistical Methods. The Iowa State College Press, Ames, Iowa, 1940.

SEROLOGICAL STUDIES ON INFLUENZA DURING A NINE-MONTH PERIOD[1]

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The anticipated influenza B epidemic materialized late in November, 1945 and reached its peak during the month of December. Sporadic cases were recognized in the United States (1) for a period of some months preceding the actual epidemic. The importance of recognizing this pre-epidemic period is unquestionably a necessity in order to avail ourselves of the prepared vaccines. It has been demonstrated (2–12) that immunization against the influenza viruses elicits an antibody increase and offers protection to the experimental inhalation of the virus strains. Francis and coworkers (13) have demonstrated further that vaccination with inactivated influenza viruses Types A and B offers protection against influenza B during an epidemic. That this epidemic was due to Type B has also been shown by Dalldorf and Rice (14).

It is recognized that other agents are responsible for certain cases of bacteria-free pneumonias. Clinically these various types of virus pneumonia often resemble each other. It is essential, therefore, to determine, if possible, the agent responsible for the infection. Their differentiation may be made by isolation of the virus or by serological studies. In many instances, failure to isolate an organism does not eliminate its possible role as causative agent. On the other hand, the development of specific antibodies in convalescent sera is one criterion that is considered to be conclusive evidence of contact with the specific antigen. Many agents have been isolated and serological technics for their identification have been developed.

These various viral technics available for laboratory diagnosis are now routinely employed in many laboratories. The examination of serum samples for the presence of antibodies to viruses, as well as those producing a clinical syndrome similar to that produced by virus agents, may be made by the Hirst test (15) for influenza; cold agglutination (16) or its modification (17), and the indifferent Streptococcus MG (18) for atypical pneumonia; and the commercial product Lygranum CF[2] (19 to 23) for members of the psittacosis group.

The experiments were designed in order (*a*) to ascertain the approximate time of the epidemics' appearance or an indication of the epidemics' approach by pre-epidemic increases in serum antibodies, (*b*) to determine the nature of the epidemic in this area, (*c*) to determine the effect of vaccination, and (*d*) to determine what antibody titers may be expected in a group of presumably normal individuals.

MATERIALS AND METHODS

Sera were derived from 2 sources. One group was obtained from 52 medical students who were bled each month for a period of 9 months from October, 1945 to June, 1946, inclusive. In this group 3 were females and the remainder were males. Of these, 15 students were given the concentrated A and B influenza vaccine prepared from chick embryos. Except for 1 member of this group, all received their vaccinations after their second blood specimen had been obtained. The lone student was vaccinated after his third blood sample had been taken. The 37 other members of this group were either members of the naval unit or civilians and were not required to be vaccinated.

A careful record of each student's health was maintained with histories obtained before the program was established and at each monthly bleeding. By coincidence, the vaccination program and the influenza epidemic were concurrent.

Another group of sera were obtained from the university student infirmary and consisted of 66 acute phase sera and 25 pairs of acute and convalescent phase sera. The donors of these sera were all university students admitted with upper respiratory infections during the

[1] Aided by a grant from the Hendricks Research Fund.

[2] (E. R. Squibb & Sons.) This antigen is used only because of its convenience, rather than its specificity. The use of a psittacosis antigen would be preferable, but more difficult to obtain.

epidemic period. In all cases, attempts were made to obtain a convalescent phase serum sample. Wherever cooperation was lacking, the acute phase sample was placed in the "single serum group" (these are discussed more fully under results). All acute phase sera were taken upon admission to the infirmary and in most cases were obtained during the first 2 days of illness. The convalescent sera were obtained 2 to 6 weeks following infection. The clinical diagnosis of this group was varied, but all were considered to be respiratory infections. The number of males and females in this group was approximately equal.

All sera were handled in an identical manner. The blood samples were obtained under sterile conditions and maintained in the sterile state. Upon receiving the blood sample, it was allowed to stand at room temperature for several hours, then centrifugated, and the serum removed. All sera were frozen and stored at −70° C. until ready to be tested. Prior to the titration of the serum, it was thawed and sufficient amounts removed and inactivated at 56° C. These were to be used in the agglutination-inhibition and complement-fixation tests. The cold agglutination and the streptococcus agglutination tests were made immediately following the thawing of the serum sample. All sera from the same individual were treated at the same time using the same test preparations.

The sera were stored in sterile vials. All titrations were completed within 24 hours after removal of the sera from the frozen state and the sera were kept at 4° C. when not in actual use.

Cold agglutination: The procedure was essentially that recommended by the Commission on Acute Respiratory Diseases (17). It consisted of adding 0.5 ml. of a 0.2 per cent suspension of fresh type O cells, obtained from the same donor throughout, to 0.5 ml. 2-fold serial serum dilutions starting at 1:5. The tubes were shaken and kept overnight at 4° C. Readings were made on the following day before the tubes had any opportunity to become warm. The end point consisted of that dilution giving definite agglutination of the cells. All titers expressed are reciprocals of the *final* dilutions of the serum.

Streptococcus MG No. 9:[3] From a culture of this organism bacterial suspensions were made and tested in the manner described by Thomas, *et al* (18). The organisms were washed 3 times in saline and a suspension corresponding approximately to a No. 3 McFarland standard was made. The streptococci were killed by heating at 60° C. for 1 hour. To 0.5 ml. serum dilution starting at 1:5, an equal volume of streptococcus suspensions was added. This mixture was placed in a 37° C. water bath for 2 hours, then overnight at 4° C., and then again at 37° C. for 2 hours. Following this, the tubes were shaken and read. The degree of agglutination used as a standard was that of the original workers. The dilution of serum giving a 1 plus reaction was used as the titer and is expressed as a *final* dilution.

Agglutination-inhibition test: The procedure used here is the same as is described by Hirst (15) except that we employed a 1 per cent chicken red cell suspension as used by Henle, *et al* (12). The virus was obtained from the allantoic fluid of 10- to 11-day-old embryos after numerous passages. The fluid from the embryos was obtained in the usual manner and then pooled. This pool was then titrated and separated into smaller amounts for storage. All virus pools were stored at −70° C. until needed. When fresh virus was required, the tube was removed at least 1 day prior to its use, thawed, and allowed to stand at 4° C. A hemagglutination titration was again made and exactly 4 units antigen were used. The pools maintained the same titer for the entire test period.

Sera were tested for inhibitory substances to the influenza viruses A, B,[4] and Swine.[5] The PR 8 and Lee strains of influenza A and B were used respectively. Readings were made after 75 minutes at room temperature and the titers expressed are those giving a 2 plus or better inhibition of agglutination. A standard red cell suspension as well as the degree of clumping was used for comparison. All sera from the same individual were titrated against the influenza strains at the same time. The saline and the red cells were dispensed with an automatic pipetting machine. To avoid possible mixing of influenza strains, the same person worked with the same strains throughout the experiment. Each virus strain had its own glassware. The expressed titers are *final* dilutions.

Henle and coworkers (12) discuss the possibility of crossing the strains, which, on the basis of Ziegler's and Horsfall's work on interference (24), appears to preclude this possibility. However, titrations in immunized mice demonstrated that our virus strains were pure.

Complement-fixation: All sera were tested for antibodies to members of the psittacosis group of viruses with Lygranum CF. To 0.2 ml. inactivated serum dilutions, 0.2 ml. complement (2 units) was added, followed by 0.2 ml. antigen (1 unit). These were placed in a 37° C. water bath for 1¼ hours and then 0.4 ml. sensitized cells were added to all tubes. These were then further incubated for ½ hour and reading made. The usual anticomplementary and non-specific controls were included in all tests. The titers of sera giving 50 per cent or better fixation were considered positive and are expressed as *initial* dilutions.

Included with all tests, *i.e.* cold agglutination, streptococcus-agglutination, agglutination-inhibition, and the Lygranum complement fixation tests, were positive serum controls.

RESULTS

Patients

An increase in admissions to the student infirmary became apparent late in November.

[3] A culture of this organism was received from Dr. Frank L. Horsfall, Jr.

[4] These two strains were supplied by Dr. Werner Henle.

[5] Dr. Gilbert Dalldorf supplied the Swine strain.

TABLE I

Incidence of influenza B among students admitted to the student infirmary with upper respiratory infections, November 1945 to February 1946

Month	Total number of admissions*	Total number of acute and convalescent sera tested	Influenza B	Incidence
				per cent
Nov.	4	1	1	4.0
Dec.	48	18	17	68.0
Jan.	8	5	2	8.0
Feb.	6	1	0	0.0
Total	66	25	20	80.0

These admissions increased rapidly during the month of December, gradually receding during the months of January and February. Table I shows the total number of students admitted into the student infirmary during this 4-month period * for acute upper respiratory infections. Of the 66 admissions, 25 pairs of acute and convalescent sera were obtained. The greatest number of admissions occurred in December with 48 cases. There were 4 cases late in November, 8 in January, and 6 in February. Of the paired sera, we obtained 1 in November, 18 in December, 5 in January, and 1 in February. Upon titration for influenza B, these were shown to have an incidence of 4.0 per cent, 68.0 per cent, 8.0 per cent, and 0.0 per cent for those months respectively. The total of 80.0 per cent shows a 4-fold or better increase in antibodies to influenza B.

The sera were divided into 2 groups for convenience, one group consisting of the paired sera, the other group composed of a single serum sample. The results obtained from the cold agglutination, indifferent streptococcus No. 9 agglutination, complement-fixation (for members of the psittacosis group) and agglutination-inhibition (for influenza A, B, and Swine) tests on the first group are shown in Table II.

Of the 25 paired sera, 3 showed increases in cold agglutinins, whereas 3 showed cold agglutinins but no increases or a drop in titer during convalescence. Of the 3 with titer increases, patients Nos. 58 and 108 were diagnosed clinically as atypical pneumonia with characteristic pulmonary involvement. Case 96, although showing a rise in cold agglutinins, demonstrated a more significant increase in influenza B antibodies. Clinically, this patient did not have any extensive pulmonary involvement and was considered to be influenza B. Patients 79, 85, and 86 all showed the presence of cold agglutinins. These titers, because of their failure to increase, were considered to be of little significance, and therefore, non-specific in nature.

TABLE II

Titers of acute and convalescent sera obtained from patients during epidemic; winter 1945–1946

Serum no.	Cold agglutination	Streptococcus MG no. 9	Psittacosis group	Influenza A	Influenza B	Influenza Swine
56 A	0	10	0	80	320	60
B	0	15	0	80	640	60
58 A	20	0	0	320	80	20
B	40	0	0	80	10	40
59 A	0	0	0	160	0	0
B	0	0	0	160	160	0
60 A	0	0	0	160	1,920	80
B	0	0	0	160	1,920	60
66 A	0	20	0	80	320	40
B	0	10	0	80	480	40
67 A	0	0	0	80	40	20
B	0	0	0	80	2,560	20
74 A	0	0	0	40	80	20
B	0	0	0	40	5,120	20
75 A	0	10	0	160	160	40
B	0	10	0	320	2,560	40
72 A	0	20	0	320	160	40
B	0	40	0	320	10,240	40
76 A	0	0	0	80	320	160
B	0	0	0	80	2,560	320
79 A	20	20	0	80	640	640
B	10	20	0	160	2,560	640
83 A	0	10	0	40	320	40
B	0	10	0	60	5,120	40
84 A	0	10	0	160	320	320
B	0	20	0	320	10,240	320
85 A	15	15	0	160	320	40
B	0	10	0	320	2,560	40
86 A	15	15	0	80	640	320
B	15	20	0	80	10,240	640
87 A	0	40	0	640	640	320
B	0	40	0	640	10,240	320
94 A	0	10	0	160	1,280	160
B	0	10	0	160	2,560	320
96 A	0	20	0	640	320	80
B	20	40	0	1,280	5,120	80
100 A	0	20	0	320	1,280	80
B	0	20	0	640	2,560	80
108 A	0	20	0	320	460	960
B	30	40	0	640	320	1,280
111 A	0	0	0	320	160	1,280
B	0	0	0	320	160	1,280
110 A	0	0	0	320	40	640
B	0	0	0	640	160	320
113 A	0	10	0	80	80	320
B	0	20	0	80	10,240	60
114 A	0	20	0	80	320	320
B	0	15	0	80	320	320
121 A	0	0	0	320	640	160
B	0	0	0	640	640	320

* Because of facilities at the infirmary, this represents only a small part of the university students. Many were entered in other hospitals.

Sixteen patients demonstrated antibodies to the indifferent streptococcus MG No. 9. Their titers ranged from 10 to 40 without any showing significant increases. Of the patients showing increases in cold agglutinins, patients 96 and 108 gave a 2-fold rise in streptococcus antibodies. Patient 58, although diagnosed as atypical pneumonia, did not have any agglutinins for this indifferent streptococcus. Many of the patients with influenza B had low titers for this organism.

In this group, there were no complement fixing antibodies to the members of the psittacosis group. Several, however, were reactive with the normal chick embryo control.

The titers for influenza A and Swine influenza showed no significant increase although several had high antibody levels. When tested against the Lee strain of influenza B, 15 patients showed 4-fold or better increases in their convalescent sera. Five patients showed only 2-fold or even no increase over their acute phase titers. However, inasmuch as the acute phase sera were obtained after several days of illness and the titers were quite high, we have considered them to be indicative of influenza B.

Of the single serum group, 7 patients demonstrated cold agglutinins in titers ranging from 10 to 60. A large number showed agglutinins for the streptococcus; and, as in the other group, they were mainly of low titer. One serum however, had a titer of 160. This titer along with a cold agglutinin titer of 60 and low influenza titers makes one strongly suspect a possible atypical pneumonia. There seemed to be little correlation between the cold agglutinin and the streptococcus titer, as in the other group.

Titrating these sera for antibodies to the psittacosis group gave 3 positives. Two of these were quite low, a 1:5 serum dilution in each case giving better than 50 per cent fixation, while one serum titered to 20.

The influenza results are the usual titers obtained when single serum samples are titrated. A wide individual variation was apparent. Several of the sera did show high titers, one giving a titer of 10,240.

These sera that had high influenza B antibodies were from students who had been ill several days before entering the infirmary. This would account for the high titers due to the rapid production of influenzal antibodies.

Many of the patients from whom only single serum samples were obtained perhaps would have demonstrated antibody increases had convalescent serum been received. In some of these, as stated above, where the acute phase specimen was taken after a few days of illness, significant titers may be noted.

Students: The sera obtained from these students were handled in the same manner as the hospitalized patients. All 9 serum samples from each student were titrated at the same time using the same test materials. Of the 52 students, 3 were unable to continue after their fourth bleeding. Their 4 samples, however, were tested.

The majority of the student serum samples had titers of less than 10 for cold agglutinins. There were a few that showed a titer of 10 and some a titer of 20. There was only 1 student with a titer of 30 and again only one had a titer of 40. No greater titers were obtained. Although there was a greater number of students with titers for the indifferent streptococcus, here, again, relatively low titers were obtained throughout. There seemed to be little correlation between the presence of cold agglutinins and streptococcal antibodies as was noted in the hospitalized group. There may have been a slight correlation between streptococcal antibodies and the presence of upper respiratory infections such as colds and sore throats, but the evidence is insufficient to warrant any definite conclusion.

With regard to the psittacosis group, there were a few sera that gave positive reactions for the group antigen in dilutions of 1:5. In 1 case, all 9 sera, *i.e.*, each monthly sample, were positive at this dilution. The other sera were positives usually for 1 or 2 months at most. These positives did not react with the normal control antigen. In a few other instances, there were some students who had sera that reacted both with the test antigen and the control antigen. These apparently were non-specific, and so were not included with our positive results.

Table III contains the results of titrating sera for antibodies to influenza A, B, and Swine. The results have been summarized on this table and show the titers of the students for each month.

Each number represents the number of students having that titer for the designated antigen in each represented month. Fifteen students in this group were vaccinated with the army vaccine against influenza A and B following their November bleeding. The first two serum samples were quite low when tested for influenza A and in the main showed very little variation during the course of the winter. It may be seen in Table III that the majority of students did not demonstrate titers greater than 320. The unvaccinated group maintained a constant titer with some students showing a 1-tube variation which was considered insignificant. Two members had no demonstrable titers for the entire period, while 5 students, although initially having no titer, developed antibodies during the course of the winter. The vaccinated group demonstrated, in most instances, at least a 4-fold antibody rise. However, 3 members of the group gave only a 2-fold rise and 2 members showed no antibody increase at all. Of those producing increased antibody levels, the levels resulting were variable with relation to the increase and duration. Several maintained constant titers for the 7 tested months following their vaccination, whereas others demonstrated antibody decreases for 1 or more months previous to their last sample.

The titers for influenza B were considerably higher in the majority of cases. Table III shows that the majority of students had antibody titers greater than 320. This was evident before the start of the vaccination program and was considered to be indicative of subclinical infection prior to the beginning of this study. Several unvaccinated students demonstrated 4-fold or better antibody rises to this virus in their December sample. None of these had reported infection or any illness during the preceding month. This would appear to indicate that they had come in contact with the virus during the epidemic without any apparent effect other than the stimulation of influenza B antibodies. As in influenza A, there are individual variations in the duration of the antibody level. Interesting is the fact that the titers of those receiving the vaccine became higher for this virus than for the Type A strain. The high initial titer, *i.e.*, the antibody level of October's serum sample, would seem to indicate that contact to the virus had been made, and makes us feel that the virus was present in the community several months prior to the actual outbreak. We were interested in observing that the general titers for *Swine influenza*, although not comparable to those for influenza B, were usually higher than those for influenza A. There appeared to be little effect resulting from the vaccination; only 3 of the 15 had more than a 2-fold antibody increase. Also, the titers of this group appeared to have very little relationship to the influenza A titers. Three members of this group demonstrated no antibody level during the entire series.

We have averaged the titers of the vaccinated and unvaccinated groups for each month. The averaged titers for each group are represented in Figure 1. It is evident that the titers for influenza

TABLE III

The monthly titers of the student group, to influenza A, B, and Swine

Following the November bleeding, 15 students received the influenza A and B vaccine.

Influenza antigen	Antibody titer (final dilution)	Month (1945-1946)								
		Oct.	Nov.	Dec.	Jan.	Feb	Mar.	Apr.	May	June
A	0	8	7	2	3	3	3	3	3	3
	40	9	9	8	6	5	5	5	5	16
	80	14	15	12	10	9	6	9	9	14
	160	13	13	17	16	18	6	19	19	14
	320	4	3	5	9	4	21	4	4	3
	640	3	4	6	5	5	5	6	6	6
	1,280	1	1	1	1	1	5	2	3	3
	2,560	0	0	1	2	2	3	1	0	0
	5,120	0	0	0	0	0	0	0	0	0
	10,240	0	0	0	0	0	0	0	0	0
B	0	2	2	0	0	0	0	0	0	0
	40	2	3	1	1	1	1	2	1	1
	80	4	2	1	2	2	2	1	2	2
	160	4	5	4	1	2	3	4	3	3
	320	8	6	4	7	8	4	4	7	9
	640	12	17	14	14	11	16	15	14	12
	1,280	10	7	9	8	8	6	6	6	6
	2,560	4	5	8	8	9	9	9	9	9
	5,120	6	5	8	7	3	4	5	5	6
	10,240	0	0	3	4	5	4	3	2	1
Swine	0	6	5	4	3	3	3	3	3	3
	40	5	5	3	1	2	2	2	2	3
	80	6	7	7	8	8	7	9	10	9
	160	15	17	17	19	17	19	17	15	17
	320	10	9	12	12	10	9	9	9	9
	640	6	5	4	3	3	3	3	5	3
	1,280	3	4	4	4	3	5	6	5	4
	2,560	1	0	1	2	3	1	0	0	1
	5,120	0	0	0	0	0	0	0	0	0
	10,240	0	0	0	0	0	0	0	0	0

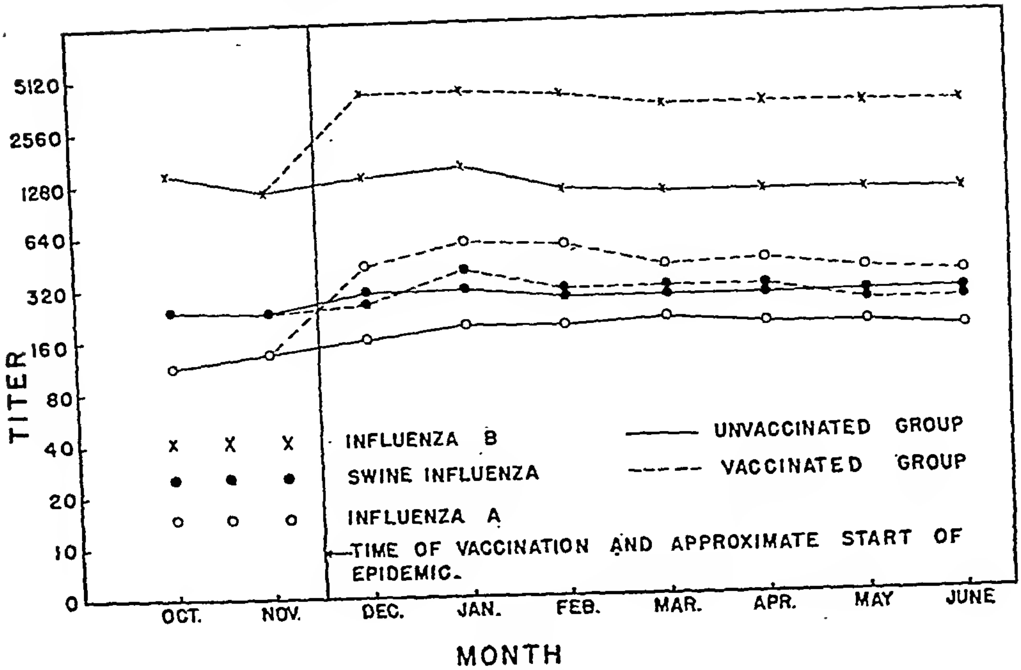

FIG. 1. THE MONTHLY TITERS OF THE VACCINATED AND UNVACCINATED STUDENTS
The points represent the average of the groups

B were higher than the other viruses, even among the unvaccinated group. The vaccinated group (represented by the broken line) subsequent to their vaccination had immediate increases in their titers. Of the 15 students receiving the vaccine, none was hospitalized, although several did report "colds." There was a slight antibody level increase among the unvaccinated group coincident with the time of vaccination. This was probably due to contacts or to cases in this group during the epidemic.

A few of the students were ill with influenza B, as is evident by the increased antibody titers seen in the serum samples following their illness. In some cases, as mentioned above, antibody increases were obtained, but without any history of illness prior to the antibody increase reported.

The individual variation to vaccination is quite apparent in several cases. For example, student 10 (Figure 2) maintained a constant titer for influenza A and Swine, of 160 throughout the test period. On the other hand, his titer for influenza B remained unchanged following his vaccination but jumped from a relatively high titer to a much higher titer 2 months after vaccination. This higher titer was maintained for a period of 5 months before dropping to its original level. Another student, No. 17, also showed no reaction to the vaccination, maintaining the same level for all 3 viruses for some time.

These titers with several other representative titrations may be seen in Figure 2. It is quite apparent that individual variation with response to vaccination and infection may be noted in immunological studies of this type.

DISCUSSION

The evidence presented indicates that the epidemic in the civilian population of this area was due to influenza B. Indications for the presence of this virus in the population previous to the actual outbreak have been mentioned (1, 13, 25), and further evidence is now reported.

The importance of recognition of the virus in advance of the epidemic cannot be stressed sufficiently. The value of vaccination during this epidemic and the importance of the agent's recognition have been stressed by Francis and coworkers (13). We can only speculate, but the use of influenza vaccines at the proper time may well have reduced our number of cases considerably. That there were many more cases among the population

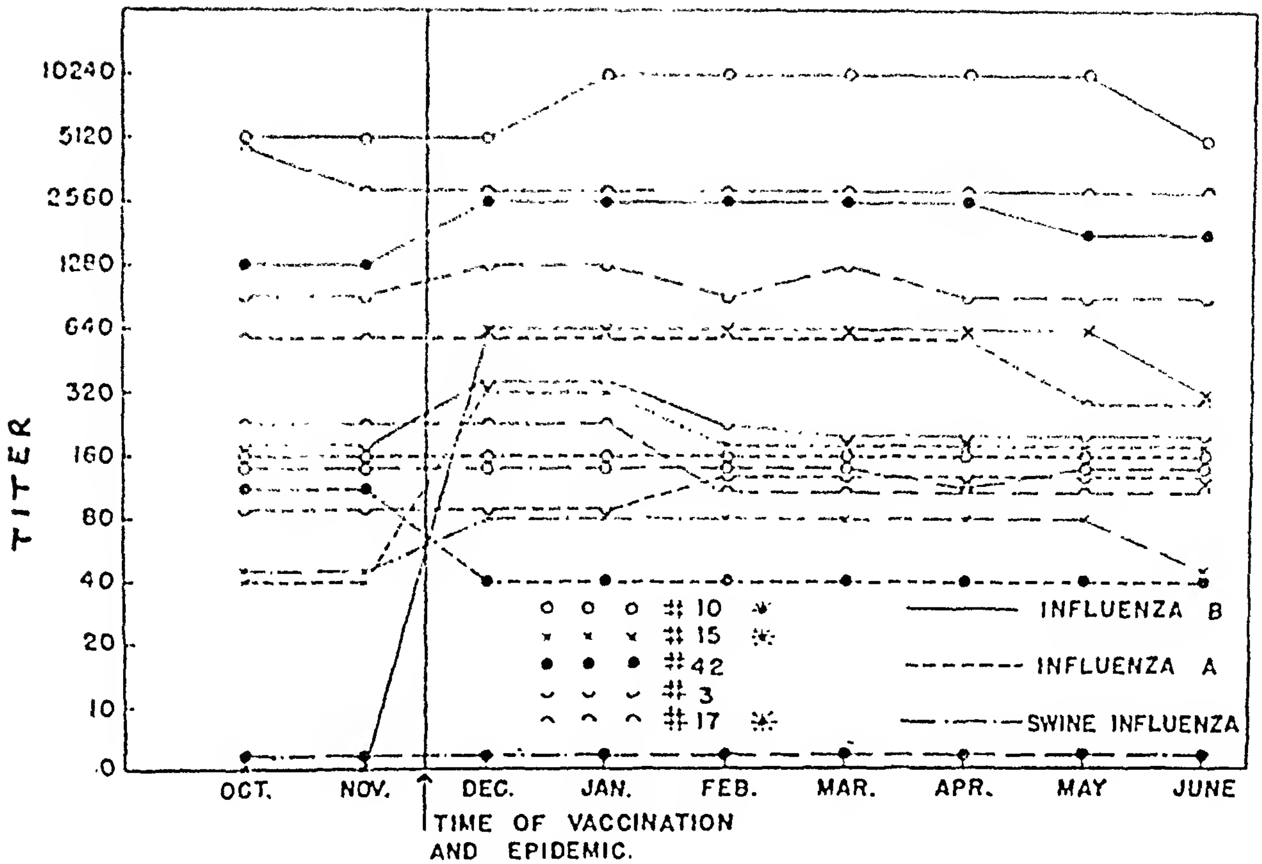

FIG. 2. INDIVIDUAL TITERS OF FIVE STUDENTS OVER THE NINE MONTH PERIOD FOR INFLUENZA A, B AND SWINE

* Vaccinated following their November bleeding.

was recognized, since the disease varies among individuals in its severity and need for hospitalization. The need for studies along these lines is imperative for the early recognition of the etiologic agent in order to administer vaccines at the proper time. The time element for administration of the vaccine is of importance as has been shown by several workers (8 to 11). Reinfection, although in a milder form, may occur after several months have elapsed between vaccination and experimental infection. This is apparent in the antibody decline following vaccination subsequent to its initial increase. The results indicate that the prophylactic administration of the influenza vaccine should be given during the month of September in order to offer the desired protection before the outbreak of the epidemic.

The significance of antibody levels is still questionable in resistance to influenza. What may be considered a high level apparently is relative to the individual. Several individuals in our groups had no initial level. This was evident among the patients and the students. There were several who had no initial titers to one or more of the influenza strains. Then, only because of vaccination or possibly infection, were titers obtained. We are still unable to determine what may be considered as a protective antibody titer to each individual.

Usually, the antibody response to infection with influenza B resulted in the production of antibodies specific for that agent. There were some individuals who developed a 2-fold antibody increase to our PR 8 strains and some to our Swine strain. We feel confident that these slight increases are not due to antibody destruction because all materials were handled in such a manner as to avoid bacterial growth. The possibility of an anamnestic reaction as postulated by Bodily and Eaton (26) must be considered. These authors also suggest that their high titers to the Swine strains may be due to the strain responsible for their cases which have a greater antigenic fraction in common with the Swine strain than

other strains of A. Another consideration may be that some individuals respond with greater antibody levels than others to the antigenic components present in the infecting strains. Henle, *et al* (12), although using sterile sera, also found similar reactions, *i.e.,* the failure to respond to vaccination among certain individuals as well as the increased antibody to the heterologous virus. These factors may be applicable to our cases, except for the usual consideration that there are no related antigenic components between influenza B and Swine. However, there is the possibility that the strain of influenza B causing the epidemic in this locality may have contained some Swine component. The failure to find significant increases to influenza A makes us feel that we are not dealing with a dual infection.

The presence of antibodies to Swine influenza in human sera has been a debatable point for some time. Whether these antibodies are resultant from previous infection with a virus of the pandemic type, as had been postulated by Shope (28), or due to repeated exposure to the human influenza virus of the pandemic type, as proposed by Francis and Magill (29), cannot be answered yet.

Our results would seem to indicate that the titers obtained for Swine influenza could be due only to infection with an agent containing Swine antigenic components. This would be possible only if infection occurred with an A strain composed of some Swine components, a Swine strain which is a variant of influenza A, or a B strain with Swine components. This last possibility is unlikely unless there is a B strain as yet unisolated or untested for the presence of common antigenic fractions with the Swine strain. If the Swine titers are due to infection with an A type, what happened to the A antibodies? Several of our group had no measurable amount of A antibody. Does the A antibody disappear before the Swine antibody? It appears that the probable explanation is that the Swine strain is a type A strain differing from the PR 8 strain in its antigenic structure.

From the results it is evident that the individuals' immunological response to these 3 influenza strains are in many instances completely independent.

With our results, we were able to ascertain many of the answers to our questions. We were somewhat disappointed in the results of the titrations inasmuch as we had anticipated a much sharper antibody increase during the epidemic. However, this was compensated by the fact that the evidence indicated that the virus for the epidemic was present far before the actual outbreak. Also, factors other than the presence of the virus are probably necessary for the outbreak. That the epidemic was due to influenza B has been discussed. There was little need for any differential diagnosis of this epidemic from other syndromes since infections by other agents did not occur to any great extent. That the method we used was satisfactory remains as yet to be determined under conditions other than those present during this study. Perhaps other agents, for example, that isolated by Meicklejohn and coworkers (30), should be tested for at the same time. At any rate, it is felt that studies along these lines would strongly indicate the etiological agent of this type of respiratory infection.

Although the vaccination occurred incidental to our original experiments and the members vaccinated are relatively few, the results of the vaccination confirm the findings of other workers in that usually strong results were obtained. The somewhat ambiguous immunological response demonstrated by a few does not appear to belittle the actual efficacy of vaccination. This seems to be in accord with the findings of other workers (12, 26). As would be expected, the antibody level of such a group would be varied. In all cases, the antibodies present were indicative of the patients' past contacts. In most cases, antibodies were present for the influenza viruses. One must also consider the possibility of other respiratory infections which are clinically similar to influenza. These may be differentiated only by the failure to produce a specific influenza antibody response and the production of an antibody to the particular etiological agent. Such agents are members of the psittacosis group (22, 31, 32, 33) and the virus of atypical pneumonia (30). Florman (23), employing Lygranum CF for studies on these agents as possible causes of virus pneumonias, suggests employment of this type of antigen for the diagnosis of agents of this group. As we have demonstrated, the presence of antibodies to the

patients with this group were few and the titers obtained could easily have been nonspecific. Several workers have reported that a low percentage of normal sera will give positive reactions with antigens of this nature when employed in low dilutions (21, 23, 34, 35). None of the students gave histories to indicate contact with members of this group. The patient who titered to 20, was First negative and unable to give a history of contact with birds. The interpretation of this titer is, therefore, difficult.

The value of cold agglutinins and streptococcal agglutination is still questionable. On the basis of our results, their value still remains an enigma. However, in agreement with Favour (35) and Finland, *et al* (37), we feel that our results demonstrate that normal individuals as well as many conditions cause type O cells to agglutinate in the cold with titers of less than 40. And still more individuals cause the agglutination of the indifferent streptococcus in low dilutions, as also observed by Finland, *et al* (38). We believe that these diagnostic procedures are of aid in atypical pneumonia only when viewed with other procedures such as x-ray and clinical findings.

It is felt that this group of tests may be of value to the laboratory in establishing the etiology of respiratory infections. Also, because of their simplicity, diagnostic laboratories should be able to employ them as routine group tests in the laboratory diagnosis.

CONCLUSIONS

1. The epidemic in this area was due to influenza B.
2. This virus was present for several months before the actual outbreak.
3. Vaccination results in an antibody increase lasting in most cases for at least 5 months. Vaccines should then be administered in the fall of the year in order to prevent infection.
4. Serological studies of this nature are of value in: (*a*) determining the etiologic agent, (*b*) ascertaining the duration of antibody titers, and (*c*) estimating the approximate time for administration of vaccines.
5. These tests may be used as a battery of tests by the laboratory, in order to ascertain the etiology of this type of respiratory infection.

BIBLIOGRAPHY

1. Reports from the Commission on Influenza to the Office of the Surgeon General, U. S. Army, Preventive Medicine Service, Washington, D. C. (Cited by Francis, *et al*, Protective effect of vaccination against epidemic influenza B. J. A. M. A., 1946, 131, 275.)
2. Francis, T., Jr., Intranasal inoculation of human individuals with the virus of epidemic influenza. Proc. Soc. Exper. Biol. & Med., 1939, 41, 537.
3. Burnet, F. M., and Foley, M., The results of intranasal inoculation of modified and unmodified influenza virus strains in human volunteers. M. J. Australia, 1940, 2, 655.
4. Eaton, M. D., and Martin, W. P., Immunization with inactive virus of influenza B. Comparison of antibody response with that produced by infection. Pub. Health Rep., 1942, 57, 433.
5. Bull, D. R., and Burnet, F. M., Experimental immunization of volunteers against influenza virus B. M. J. Australia, 1943, 1, 389.
6. Henle, W., Henle, G., and Stokes, J., Jr., Demonstration of the efficacy of vaccination against influenza type A by experimental infection of human beings. J. Immunol., 1943, 46, 163.
7. Francis, T., Jr., Pearson, H. E., Salk, J. E., and Brown, P. N., Immunity in human subjects artificially infected with influenza virus, type B. Am. J. Pub. Health, 1944, 34, 317.
8. Personnel of Naval Laboratory Research Unit No. 1, Experimental human influenza. Am. J. M. Sc., 1944, 207, 306.
9. Hirst, G. K., Rickard, E. R., and Friedewald, W. F., Studies in human immunization against influenza. Duration of immunity induced by inactive virus. J. Exper. Med., 1944, 80, 265.
10. Francis, T., Jr., Salk, J. E., Pearson, H. E., and Brown, P. N., Protective effect of vaccination against induced influenza A. J. Clin. Invest., 1945, 24, 536.
11. Salk, J. E., Pearson, H. E., Brown, P. N., and Francis, T., Jr., Protective effect of vaccination against induced influenza B. J. Clin. Invest., 1945, 24, 547.
12. Henle, W., Henle, G., Stokes, J., Jr., and Maris, E. P., Experimental exposure of human subjects to viruses of influenza. J. Immunol., 1946, 52, 145.
13. Francis, T., Jr., Salk, J. E., and Brace, W. M., The protective effect of vaccination against epidemic influenza B. J. A. M. A., 1946, 131, 275.
14. Dalldorf, G., and Rice, G. E., A preliminary state wide influenza survey using routine blood specimens. (In press.)
15. Hirst, G. K., The quantitative determination of influenza virus and antibodies by means of red cell agglutination. J. Exper. Med., 1942, 75, 49.
16. Peterson, O. L., Ham, T. H., and Finland, M., Cold agglutinins (antihemagglutinins) in primary atypical pneumonias. Science, 1943, 97, 167.

17. Commission on Acute Respiratory Diseases, Cold hemagglutinins in primary atypical pneumonia and other respiratory infections. Am. J. M. Sc., 1944, 208, 742.
18. Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., Jr., and Horsfall, F. L., Jr., Studies on primary atypical pneumonia. II. Observations concerning the relationship of a non-hemolytic streptococcus to the disease. J. Clin. Invest., 1945, 24, 227.
19. Eaton, M. D., and Corey, M., Complement fixation in human pneumonitis with group-reactive virus antigens. Proc. Soc. Exper. Biol. & Med., 1942, 51, 165.
20. Smadel, J. E., Wertman, K., and Reagan, R. L., Yolksac complement fixation antigen for use in psittacosis-lymphogranuloma venereum group of diseases. Proc. Soc. Exper. Biol. & Med., 1943, 54, 70.
21. Rake, G., Shaffer, M. F., Jones, H. P., and McKee, C. M., Lymphogranuloma Venereum. "A monograph," E. R. Squibb and Sons, N. Y., 1943.
22. Eaton, M. D., Martin, W. P., and Beck, M. D., The antigenic relationship of the virus of meningopneumonitis and lymphogranuloma venereum. J. Exper. Med., 1945, 75, 21.
23. Florman, A. L., The use of a commercially available complement-fixing antigen for the diagnosis of elementary body types of viral infection. J. Immunol., 1945, 51, 29.
24. Ziegler, J. E., Jr., and Horsfall, F. L., Jr., Interference between the influenza viruses. I. The effect of active virus upon the multiplication of influenza viruses in the chick embryo. J. Exper. Med., 1944, 79, 361.
25. Salk, J. E., Influenza in Europe during the winter of 1945–46. J. Bact., 1946, 51, 632.
26. Bodily, H. L., and Eaton, M. D., Specificity of the antibody response of human beings to strains of influenza virus. J. Immunol., 1942, 45, 193.
27. Lennette, E. H., Rickard, E. R., Hirst, G. K., and Horsfall, F. L., Jr., The diverse etiology of epidemic influenza. Pub. Health Rep., 1941, 56, 1777.
28. Shope, R. E., The incidence of neutralizing antibodies for swine influenza virus in the sera of human beings of different ages. J. Exper. Med., 1936, 63, 669.
29. Francis, T., Jr., and Magill, T. P., The incidence of neutralizing antibodies for human influenza virus in the serum of human individuals of different ages. J. Exper. Med., 1936, 63, 655.
30. Meiklejohn, G., Eaton, M. D., and Van Herick, W., A clinical report on cases of primary atypical pneumonia caused by a new virus. J. Clin. Invest., 1945, 24, 241.
31. Smadel, J. E., Atypical pneunonia and psittacosis. J. Clin. Invest., 1943, 22, 57.
32. Eaton, M. D., Beck, M. D., and Pearson, H. E., A virus from cases of atypical pneunonia. Relation to the virus of meningopneumonitis and psittacosis. J. Exper. Med., 1941, 73, 641.
33. Rake, G., Eaton, M. D., and Shaffer, M. F., Similarities and possible relationships among viruses of psittacosis, meningopneumonitis, and lymphogranuloma venereum. Proc. Soc. Exper. Biol. & Med., 1941, 48, 528.
34. Dingle, J. H., Abernethy T. J., Badger, G. F., Buddingh, G. J., Feller, A. E., Langmuir, A. D., Ruegsiggir, J. M., and Wood, B., Jr., Primary atypical pneunonia, etiology unknown (Part III). Am. J. Hyg., 1944, 39, 269.
35. Blair, J. E., The complement fixation reaction with the antigen of lymphogranuloma venereum. J. Immunol., 1944, 49, 63.
36. Favour, C. B., Autohemagglutinins—"cold agglutinins." J. Clin. Invest., 1944, 23, 891.
37. Finland, M., Peterson, O. L., Allen, H. E., Samper, B. A., and Barnes, M. W. (with the technical assistance of Muriel B. Stone), Cold agglutinins. I. Occurrence of cold isohemagglutinins in various conditions. J. Clin. Invest., 1945, 24, 451.
38. Finland, M., Samper, B. A., and Barnes, M. W., Cold agglutinins. VI. Agglutinins for an indifferent streptococcus in primary atypical pneumonia and in other conditions and their relation to cold isohemagglutinins. J. Clin. Invest., 1945, 24, 497.

COMPARATIVE ACTION OF ACETYL-BETA-METHYL CHOLINE AND HISTAMINE ON THE RESPIRATORY TRACT IN NORMALS, PATIENTS WITH HAY FEVER, AND SUBJECTS WITH BRONCHIAL ASTHMA [1]

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In a recent study (1), it was reported that the parenteral administration of histamine produced a sizable reduction of vital capacity in 8 of 9 actively asthmatic subjects, whereas it produced no significant change of vital capacity in 2 control groups of normal individuals and patients with hay fever. Furthermore, the effectiveness of histamine in producing a reduction in vital capacity varied in different asthmatic subjects and also in the same subject with the degree of asthma present at the time the tests were done. The present communication reports the action of acetyl-beta-methyl choline chloride (mecholyl chloride) on the vital capacity in asthmatic subjects, in patients with hay fever, and in a group of normal individuals and presents a comparison of the effect of mecholyl chloride with that of histamine. These assays were carried out in the hope of further elucidating the underlying mechanisms in asthmatic attacks.

In 1912, Barker and Sladen (2) noted the action of pilocarpine and atropine in bronchial asthma. Alexander and Paddock (3) induced "asthmatic-like" attacks in 10 of 20 patients with asthma by the subcutaneous injection of 3 mgm. pilocarpine. The latter workers observed that the systemic response to pilocarpine, as manifested by salivation, sweating, epiphora, flushing, and a sensation of warmth, was slightly greater in the asthmatic subjects than in the normal subjects. Starr, Elsom, Reisinger, and Richards (4) with the subcutaneous administration of 20 mgm. acetyl-beta-methyl choline inadvertently precipitated an "asthmatic-like" attack lasting 3 minutes in a young subject with a past history of asthma. Starr (5) also produced mild "asthmatic-like" attacks in asthmatic patients with acetyl-beta-methyl choline chloride given orally. Villaret and his coworkers (6) gave 20 to 40 mgm. doses of acetyl-beta-methyl choline subcutaneously to 15 asthmatic patients and produced in all of them "asthmatic-like" attacks which were regularly relieved by atropine. Moll (7) injected 5 to 20 mgm. doses of acetyl-beta-methyl choline subcutaneously in 28 asthmatic subjects and produced in 23 cases "asthmatic-like" attacks of varying intensity. The extra-pulmonary response to the drug in the asthmatic group did not differ from the response in the normal control subjects. Moll therefore made the suggestion that the peculiar tropism of choline derivatives for the lungs in asthmatic subjects was due to a local sensitivity rather than to a generalized state of increased sensitivity. Lung damage was considered an essential factor in determining the abnormal bronchial response. Dautrebande and Philippot (8) induced "asthmatic-like" attacks with aerosols of carbamino choline and studied the effectiveness of aerosols of phenyl amino propane in relieving these attacks. Ellis and Weiss (9) and Carmichael and Fraser (10) noted that when acetyl choline was given intravenously to normal subjects a sensation of substernal constriction was produced which was occasionally associated with coughing. Hurtado and Kaltreider (11) recorded a definite decrease in vital capacity associated with a sensation of substernal constriction and difficult breathing in 2 normal subjects following the intramuscular injection of acetyl-beta-methyl choline in doses of 30 mgm. and 15 mgm. respectively. Eppinger and Hess (12) in 1917 cited asthma as an example of pathologically increased vagotonia. One of the difficulties in accepting this theory of the etiology of asthma is the failure of atropine to give marked relief in the disease. Thus, while we have ob-

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served notable improvement both in symptoms and in the vital capacity in asthmatic subjects following adequate doses of atropine sulfate intravenously, there is no question that the relief is not so dramatic as that after epinephrine. While there has been no extensive study of blood cholinesterase levels in asthmatic patients, the few that have been determined have been within the normal range (13). More important than blood levels, however, would be a study of lung cholinesterase, since, if there is an excess liberation of acetyl choline in the lungs of the asthmatic subjects, one might expect an increased amount of cholinesterase to be present.

MATERIALS AND METHODS

Acetyl-beta-methyl choline, hereafter referred to as mecholyl chloride,[2] was administered to 3 groups of subjects, and the reaction of the respiratory tract was measured chiefly by recording the vital capacity by a technic already described (1). The first group was composed of 10 normal subjects who had no recognizable symptoms or manifestations of allergy. The second group included 11 patients with seasonal hay fever who had neither a history nor signs or symptoms of asthma. These latter patients were also tested in the same way after the parenteral administration of histamine. The third group was composed of 27 asthmatic subjects, the majority of whom had mild continuing asthma. However, a few patients were included who had a history of typical asthmatic attacks in the past but who had had no recent signs or symptoms of asthma. As in the previous study, an attempt was made to secure young, cooperative subjects with vital capacities not subject to unaccountable variations. In all of these subjects the action of parenteral histamine on the vital capacity was also studied for purposes of comparison. Patient B. R., a 28-year-old, single female with mild chronic asthma, was again very cooperative and repeated studies were made with her assistance.

Mecholyl chloride was dissolved in sterile physiological saline to a concentration of 20 mgm. per ml. for intramuscular injection and further diluted with sterile physiological saline to a concentration of 0.1 mgm. per ml. for intravenous administration. Care was exercised in the preparation of the solutions because of the marked hygroscopic property of the salt. The solution was placed in a sterile rubber capped bottle which was stored in a refrigerator when not in use. A fresh solution was prepared at 2- to 3-week intervals. Unless otherwise stated the drug was injected in the deltoid muscle. In some of the asthmatic group the reaction to the intravenous administration and nebulization of the drug was also observed. The maximum dose employed was 6 mgm. because experience showed that with the patients in the sitting position larger doses sometimes produced untoward systemic effects, such as weakness and dizziness, which interfered with the performance of the vital capacity test. In the asthmatic group when wheezing or respiratory discomfort was present, doses of 0.25 to 0.50 mgm. were first administered to determine the reactiveness of the patient. The dosage was then increased up to 6 mgm. or until a significant reduction in the vital capacity was produced. The intravenous dose ranged from 0.005 mgm. to 0.6 mgm. For nebulization a 1:40,000 solution was used. The degree of flushing, salivation, sweating, and tachycardia which followed the administration of mecholyl chloride was also noted. Histamine acid phosphate,[3] with 1 ml. equivalent to 0.2 mgm. histamine base, was given intravenously to the groups with hay fever and asthma in doses equivalent to 0.01 to 0.04 mgm. histamine base, except in a few instances when the drug was given intramuscularly. In the latter cases the dosage was increased.

[2] Merck & Co., Inc.

[3] Eli Lilly Company.

RESULTS AND COMMENTS

Intramuscular mecholyl chloride

NORMAL SUBJECTS: In 3 of 10 normal individuals a dose of 6 mgm. mecholyl chloride caused a definite though slight reduction in the vital capacity measuring 4 per cent, 4 per cent, and 6 per cent respectively (Table I). The tests were repeated in these 3 subjects within a few days and similar effects were obtained. All of the normal subjects experienced a sensation of substernal constriction with difficulty in respiration. In spite of this, however, 3 subjects showed a slight increase in the vital capacity after the drug was administered. It is likely that normal individuals have a slight but variable sensitivity of the respiratory tract to

TABLE I

The effect of 6 mgm. mecholyl chloride given intramuscularly on the vital capacity in normal subjects

Subject	Age	Sex	Vital capacity: Before drug	Vital capacity: Change
			ml.	*per cent*
S. L.	30	M	4,671	−1
S. K.	30	M	4,494	−0.9
W. H.	21	F	3,511	+5
J. S.	25	M	4,723	−0.9
P. K.	31	M	3,605	+6
J. C.	32	M	4,347	−0.5
R. S.	22	M	4,681	−4
			4,618	−6
R. B.	28	M	3,929	−4
			4,096	−3
W.G.	23	M	4,891	−6
			4,901	−5
R. D.	29	M	4,012	+6

mecholyl and that, if the dosage is sufficiently increased, a reduction in vital capacity might be produced in all, as is suggested by the studies of Hurtado and Kaltreider (11). However, in a subject with mild chronic bronchitis who had no allergic tendencies, the administration of 6 mgm. mecholyl chloride produced a pronounced decrease in vital capacity. Therefore it was important to make certain that the normal group had no demonstrable respiratory tract disease. In the earlier study (1), among the 10 normal subjects given 0.03 mgm. histamine base intravenously, only 1 subject had a reduction in vital capacity of 3 per cent, 2 had a reduction of 2 per cent and 2 a reduction of 1 per cent. In the remainder the vital capacity increased following the administration of the drug.

TABLE II

The effect of mecholyl chloride and histamine base on the vital capacity of subjects with hay fever and no asthma

Subject	Age	Sex	Drug administered: Mecholyl chloride I.M.	Drug administered: Histamine base I.V.	Vital capacity: Before drug	Vital capacity: Change
			mgm.	*mgm.*	*ml.*	*per cent*
L. H.	48	F	6		2,748	−2
				0.03	2,780	+3
H. W.	28	M	6		4,514	−1
				0.03	4,504	+.04
J. U.	24	M	6		4,786	−6
				0.03	4,765	−4
H. A.	24	M	6		4,264	−3
				0.03	4,494	+0.4
E. B.	23	M	6		3,981	−9
				0.03	3,950	−7
W. G.	25	M	6		4,608	−8
				0.03	4,608	−2
F. R.	23	M	6		3,961	−2
				0.03	4,055	−0.2
V. N.	18	M	6		4,138	−5
				0.03	4,190	0
L. N.	33	M	6		4,514	−8
				0.03	4,514	−9
A. H.	23	F	6		3,145	−10
				0.02	3,260	−4
L. P.	31	M	6		3,835	−10
				0.02	3,919	−4

Hay fever: In the group of 11 patients with hay fever who had neither a history nor signs or symptoms of asthma, the intramuscular injection of 6 mgm. mecholyl chloride produced some decrease in vital capacity in all of the subjects (Table II). Seven had a decrease in vital capacity of 5 per cent or greater. In comparison the intravenous administration of 0.02 to 0.03 mgm. histamine base in this same group produced a decrease in vital capacity in 6 of the 11 subjects and in only 2 instances was the decrease greater than 5 per cent. It is difficult to evaluate changes in the vital capacity of less than 5 per cent since variations of this degree may occur in the resting vital capacities. Nevertheless mecholyl chloride in the dosage used was more effective than histamine in reducing the vital capacity in these subjects. The extrapulmonary response was not different in the hay fever group than in the normal group and in some instances the extrapulmonary reaction was waning as the respiratory tract reaction was increasing. The majority of these tests were performed during the pollen season although none of the subjects complained of symptoms at the time of the tests. This may be important, since Brown and his coworkers (14) have shown that patients with hay fever and no history of bronchial asthma may frequently show a diminished vital capacity during their pollen season. It would appear worthwhile to repeat the tests on the hay fever subjects when the pollen season is terminated. Whether the more reactive patients with hay fever are those who may in later years develop asthma is a matter for conjecture. An attempt to correlate the personal and familial allergic background of a larger group of these patients with the degree of respiratory sensitivity to mecholyl chloride and histamine is planned.

Bronchial asthma: In the group of 27 asthmatic subjects, all had a reduction in vital capacity after the intramuscular administration of from 1 to 6 mgm. mecholyl chloride, and in only 1 instance was this reduction less than 5 per cent. In this same group 22 of the 27 patients had a reduction in vital capacity after parenteral histamine and in only 1 of the reactive cases was the reduction less than 5 per cent. The pattern of response to mecholyl chloride was similar in all the reactive patients although the sensitivity varied greatly from patient to patient and in the same patient varied at different times, depending on the degree of asthma present when the test was performed.

As a rule, in patients with only occasional mild attacks of asthma or in those who had only a history of asthma, the respiratory tract was less reactive to mecholyl than in subjects with active bronchial asthma. Following the intramuscular administration of an effective dose of mecholyl

chloride the vital capacity was reduced when measured 2 minutes later. At the 4-minute period in many instances and especially if the reaction was pronounced, a further decrease in vital capacity was found. At 6 minutes usually there was a return toward the resting levels in the vital capacity except in those cases when the reaction was severe. Depending then on the severity of the reaction, the vital capacity returned to the resting levels in from 10 to 30 minutes. For example, in patient B. R. after the resting vital capacities were determined, a dose of 0.5 mgm. mecholyl chloride was injected into the deltoid muscle. Two minutes later the vital capacity measured 1,881 ml., a fall of 1,233 ml. from the resting level of 3,114 ml. Four minutes after the injection the vital capacity was 1,787 ml., and at the 6-minute period measured 2,080 ml. Vital capacities were recorded at the 10-minute, 15-minute, and 20-minute periods and measured 2,727 ml., 2,968 ml., and 3,114 ml. respectively. The flushing, sensation of warmth, sweating, and salivation produced by the administration of the drug were minimal, but respirations were increased with audible inspiratory and expiratory wheezing. Subject J. H., on the other hand, had a resting vital capacity of 5,454 ml. and 2 minutes after the intramuscular injection of 4 mgm. mecholyl chloride the vital capacity measured 5,298 ml. Vital capacities were repeated at 4 minutes, 6 minutes, 10 minutes, and 20 minutes after the injection and measured 5,026 ml., 2,016 ml., 5,141 ml., and 5,507 ml. respectively.

When increasing amounts of mecholyl chloride were injected intramuscularly an increased response occurred, the vital capacity was further reduced, and the subjective symptoms were increased. In subject P. M., for example, after the resting vital capacities were determined, varied amounts of mecholyl chloride were administered by the intramuscular route. In order to eliminate the possibility of a cumulative effect of the drug, the doses were given at 20-minute intervals in the following order, 2 mgm., 4 mgm., 6 mgm., 3 mgm., and 5 mgm. Vital capacities were measured at the usual intervals of 2 minutes, 4 minutes, 6 minutes, 10 minutes, and 20 minutes. The vital capacities recorded 4 minutes after each injection of the drug showed an increased reduction with increasing dosage of mecholyl chloride (Figure 1). When identical doses of mecholyl chloride were

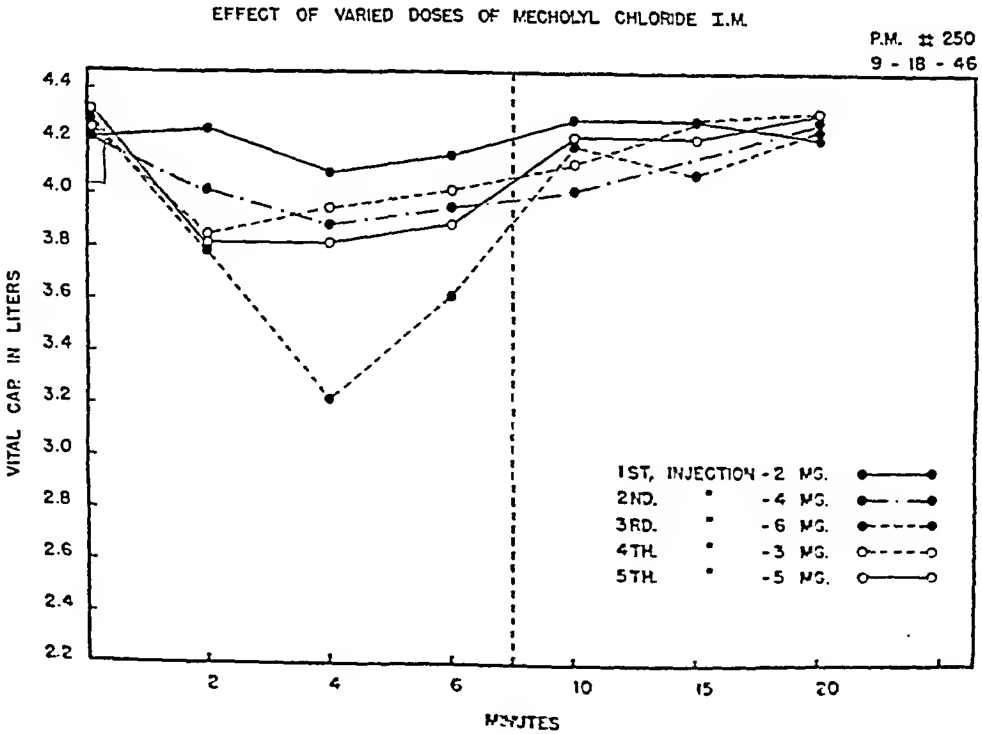

FIG. 1

injected intramuscularly at 30-minute intervals, the decrease in vital capacity which resulted in some cases varied as much as 500 ml. In subject B. R., for example, 5 intramuscular injections of 0.4 mgm. mecholyl chloride at 30-minute intervals resulted in a decreased vital capacity of 846 ml., 857 ml., 1,129 ml., 773 ml., and 658 ml. respectively from a resting level of 3,385 ml. It is possible that these variations may be due to slight differences in the site of injection of the drug in the deltoid area since we have observed a difference in the reaction pattern when the drug is given subcutaneously. There is also suggestive evidence that with a less reactive subject and with larger doses of mecholyl chloride, from 4 to 6 mgm., used, a more uniform reduction in vital capacity is obtained with repeated identical doses of the drug. In a few instances when prostigmin methyl sulfate, in doses of 0.5 to 0.75 mgm., was administered, the effect of mecholyl chloride on the respiratory tract was markedly enhanced. In view of the severe reactions that we produced in actively asthmatic subjects with small doses of intramuscular mecholyl chloride, it is difficult to understand how the subjects given 20 to 40 mgm. mecholyl chloride subcutaneously by Villaret and his coworkers were able to tolerate the reaction. It is possible that the patients were suffering only mild bronchial asthma and that the difference in the site of administration of the drug and the supine position resulted in a lessened response to drug.

In the asthmatic group we were unable to detect any notable difference from the control groups in the extrapulmonary response to mecholyl chloride, as manifested by flushing, salivation, sensation of warmth, or tachycardia. These findings confirm those reported by Moll. However, the occurrence of an increased tracheobronchial reaction to mecholyl chloride in 3 normal patients and many of our hay fever control group and some asthmatic subjects without any obvious respiratory infection would seem to indicate that lung damage was not necessarily an essential factor in determining the abnormal bronchial response as suggested by the same author. While the precise cause of this abnormal tracheobronchial response in asthmatic subjects is not clear, studies are in progress which may throw some light on the mechanism. In one individual, S. G., a very mild asthmatic subject, 6 mgm. mecholyl chloride intramuscularly provoked an attack of uncontrolled auricular fibrillation, confirmed by electrocardiogram, which subsided spontaneously in 1 hour.

Comparison of the effect on the respiratory tract of mecholyl chloride and histamine given parenterally

Since there appeared to be a difference in the response of the tracheobronchial tree to histamine and mecholyl, it appeared worthwhile to ascertain that they acted independently. While it is generally agreed that the pharmacodynamic activity of the 2 drugs in the lungs differs, it seemed possible that in the deranged state of hyperresponsiveness of the respiratory tract one substance might act to some extent through liberation of the other. There also appears to be some relationship between histamine and mecholyl chloride in that antihistamine drugs have some degree of antiacetyl choline activity and antiacetyl choline drugs have some antihistamine activity. Therefore, in patient J. D., 0.02 mgm. histamine base was administered intravenously, and a reduction of 1,944 ml. in the vital capacity followed. When the vital capacity had returned to the resting levels, 1 mgm. mecholyl chloride was given intramuscularly and a reduction of 1,234 ml. resulted. After the vital capacity had again returned to the resting levels, a dose of 30 mgm. benadryl, a potent antihistamine agent (15), was given intravenously. When a dose of 0.02 mgm. histamine base, given intravenously, was now repeated a decrease in vital capacity of only 168 ml. occurred, whereas 1 mgm. mecholyl chloride intramuscularly caused a decrease of 1,348 ml. Benadryl, therefore, afforded remarkable protection against the reduction in vital capacity due to histamine but gave no protection against the reduction due to mecholyl chloride. In patient B. R., after 30 mgm. benadryl had been given by vein and marked histamine protection resulted, a dose of 2 mgm. mecholyl chloride produced a pronounced reduction in vital capacity. In another experiment, in patient J. D., the reduction in vital capacity due to 0.01 mgm. histamine given intravenously was 481 ml. A dose of 0.75 mgm. prostigmin methyl sulfate was administered intramuscularly and a repeat injection of 0.01 mgm. histamine 20 minutes later produced a drop in vital capacity of 481 ml. Prostigmin did not augment the response to histamine. It appeared, therefore, that in the asth-

TABLE III

Effect of mecholyl chloride and histamine base on the vital capacity of patients with moderately active asthma

Subject	Age	Sex	Drug administered: Mecholyl chloride I.M.	Drug administered: Histamine base I.V.	Vital capacity: Before drug	Vital capacity: Change
			mgm.	*mgm.*	*ml.*	*per cent*
B. R.	28	F	2		3,365	−76
				0.04	3,334	−78
A. L.	33	M	4		4,504	−45
				0.03	4,901	−44
J. D.	16	F	1		3,103	−46
				0.02	3,083	−63
M. M.	38	F	4		3,396	−18
				0.03	3,322	−16
E. C.	13	F	3		1,787	−51
				0.01	1,891	−29
F. G.	29	M	4		4,159	−61
				0.03	4,107	−9
V. B.	49	M	atomized		3,961	−29
				0.02	3,793	−50
S. W.	22	F	2		3,083	−65
				0.01	3,752	−54
R. B.	19	M	2		2,989	−54
				0.02	3,783	−47
P. McD.	20	M	6		4,274	−25
				0.02	4,316	−6
W. J.	14	M	2		2,278	−37
				0.01	2,236	−29
R. B.	25	F	2		2,404	−48
				0.02	2,435	−3
I. S.	20	F	1		2,351	−80
				0.01	2,341	−86
V. C.	25	F	2		2,153	−18
				0.02	2,905	−14
S. M.	37	M	4		3,177	−25
				I.M. 0.12	3,322	−19
J. D.	19	M	3		4,389	−25
				I.M. 0.20	4,243	−44

matic individual the 2 drugs, histamine and mecholyl chloride, operated independently in producing a decrease in vital capacity.

The subjective response to both drugs in regard to the action on the respiratory tract in the asthmatic group was similar when the reaction was pronounced. The majority of subjects were unable to distinguish between them except that the mecholyl chloride response was less apt to disappear quickly. When the reaction was mild, however, the majority of subjects felt that the mecholyl chloride response was "more like a true asthmatic attack" than the histamine response.

In all patients with active bronchial asthma the respiratory tract was hyperreactive to both drugs; but, in the case of R. B., an active asthmatic, the degree of reduction in vital capacity from a dose of 0.02 mgm. histamine intravenously was only 3 per cent contrasted to a reduction in vital capacity of 48 per cent after the intramuscular injection of 2 mgm. mecholyl chloride. It appeared that the tracheobronchial tree was more reactive to mecholyl chloride than to histamine, since small doses of mecholyl chloride from 0.25 to 2.0 mgm. intramuscularly produced regularly a pronounced decrease in vital capacity (Table III).

In the group of asthmatics with only mild occasional attacks or with only a history of asthma, usually in childhood, sensitivity of the respiratory tract to mecholyl chloride was definitely more marked than sensitivity to histamine and sensitivity to both drugs was less than in the former group (Table IV). One patient, W. B., who had a history of asthma was not very reactive to either drug. A dose of 6 mgm. mecholyl chloride in this patient caused a reduction in vital capacity of only 3 per cent and the intravenous administration of 0.03 mgm. histamine was followed by a vital capacity greater than the resting value. In another patient, W. F., who had a history of typical asthma, an intramuscular dose of 6 mgm. mecholyl chloride caused a reduction of 31 per cent in the vital ca-

TABLE IV

Effect of mecholyl chloride and histamine base on the vital capacity of patients with mild, occasional bronchial asthma or a past history of asthma

Subject	Age	Sex	Drug administered: Mecholyl chloride I.M.	Drug administered: Histamine base I.V.	Vital capacity: Before drug	Vital capacity: Change
			mgm.	*mgm.*	*ml.*	*per cent*
I. B.	26	M	4		3,375	−25
				I.M. 0.20	3,428	−9
M. S.	45	F	3		3,250	−37
				0.02	2,654	−19
D. D.	43	F	6		2,581	−10
				0.03	2,529	+1
W. B.	27	M	6		4,399	−3
				0.03	4,430	+2
H. D.	22	F	6		3,125	−5
				0.03	3,240	−5
R. M.	22	F	6		3,522	−18
				0.03	3,511	−10
C. S.	26	M	4		4,180	−12
				0.03	4,358	0
S. G.	21	F	6		2,696	−18
				0.03	2,518	+3
W. F.	27	M	6		4,691	−31
				0.03	4,807	−24
G. M.	34	M	6		4,452	−15
				0.02	4,201	+1
G. C.	37	M	6		4,698	−11
				0.02	4,494	−11

pacity and 0.03 mgm. histamine base intravenously caused a reduction of 24 per cent. In 3 subjects with only mild occasional attacks of asthma, D. D., C. S., and S. G., mecholyl chloride caused significant decreases in vital capacity, whereas the intravenous administration of 0.03 mgm. histamine produced no decrease in vital capacity. In view of these findings it would appear that attention should be turned more towards discovering anticholinergic agents rather than antihistamine drugs for the treatment of bronchial asthma.

Since there is a possibility that respiratory hyperresponsiveness in the hay fever group may indicate those individuals who are potential asthmatics and since the differences in reaction of the asthmatic group to histamine and mecholyl chloride may serve to differentiate various types of asthma, it is planned to enlarge the group and attempt to correlate the reaction to the 2 drugs with the various clinical characteristics of the allergic state.

Intravenous mecholyl chloride

When mecholyl chloride was administered intravenously to asthmatic subjects, in doses of 0.005 to 0.6 mgm., the maximal reduction in vital capacity was noted in about 30 seconds and the vital capacity returned to the resting levels in 15 to 30 minutes. As might be expected with the larger doses a more pronounced reduction in vital capacity occurred and a longer time was required before the vital capacity returned to the resting levels. Thus, in subject B. R., the intravenous injection of 0.01 mgm. mecholyl chloride produced at the 30-second period a decrease of 760 ml. in the vital capacity, from the resting level of 3,170 ml. to 2,410 ml. Repeated vital capacities recorded at the 1.5, 3, 5, and 10-minute periods after the injection measured 2,510 ml., 2,770 ml., 2,950 ml., and 3,350 ml. respectively. In patient W. G., on the other hand, a dose of 0.6 mgm. mecholyl chloride given by vein produced a reduction of 2,408 ml. in the vital capacity from the resting level of 3,598 ml. to 1,087 ml. in 30 seconds after the injection. Repeated vital capacities were recorded at 1.5, 3.5, 5, 10, 20, 30, and 40-minute periods and measured 1,964 ml., 2,341 ml., 2,623 ml., 2,748 ml., 2,947 ml., 3,322 ml., and 3,469 ml. respectively. The intravenous injection of small amounts of mecholyl chloride produced a much greater reaction in the respiratory tract than much larger doses of the drug administered intramuscularly. Thus, in subject B. R., the intravenous injection of 0.1 mgm. mecholyl chloride caused a reduction of 1,800 ml. in the vital capacity as compared to a reduction of 310 ml. produced by the intramuscular injection of 0.25 mgm. of the drug. When an increased dose of mecholyl chloride is given intravenously, a greater reduction in vital capacity occurs. For example, in subject B. R., the administration of 0.01 mgm. mecholyl chloride intravenously caused a reduction of 762 ml. in the vital capacity, and, when subsequent doses of 0.03 mgm., 0.05 mgm., 0.08 mgm., and 0.10 mgm. of the drug were injected at 20-minute intervals, the following reductions in vital capacity were measured: 869 ml., 835 ml., 1,170 ml., and 1,798 ml. respectively (Figure 2). It may be seen that the 0.01 mgm., 0.03 mgm. and 0.05 mgm. doses produced a similar decrease in vital capacity but with larger doses notable increased reduction in vital capacity was observed. This same lack of sensitivity to small changes in dosage was brought out when increased doses of mecholyl chloride varying by only .005 and .01 mgm. were injected intravenously and but slight variances in reduction of the vital capacity were recorded.

Nebulized mecholyl chloride

Nebulization of a 1 : 40,000 solution of mecholyl chloride produced a profound decrease in vital capacity which suggested that perhaps the respiratory tract is more reactive to the drug when it is administered in this manner. For example, in patient B. R., 4 inhalations from the nebulizer of mecholyl chloride produced a decrease in vital capacity of 2,111 ml. from a resting level of 2,769 ml. to 658 ml. There was no evidence over a period of 5 minutes that the attack was subsiding and 0.85 mgm. atropine sulfate was given by vein with only partial relief and an increase in the vital capacity to 1,943 ml. Five minutes after the administration of 0.3 mgm. 1 : 1,000 epinephrine hydrochloride intramuscularly there was marked subjective relief and the vital capacity measured 3,344 ml. It is interesting to note that in this case the relief afforded by atropine sulfate was not dramatic.

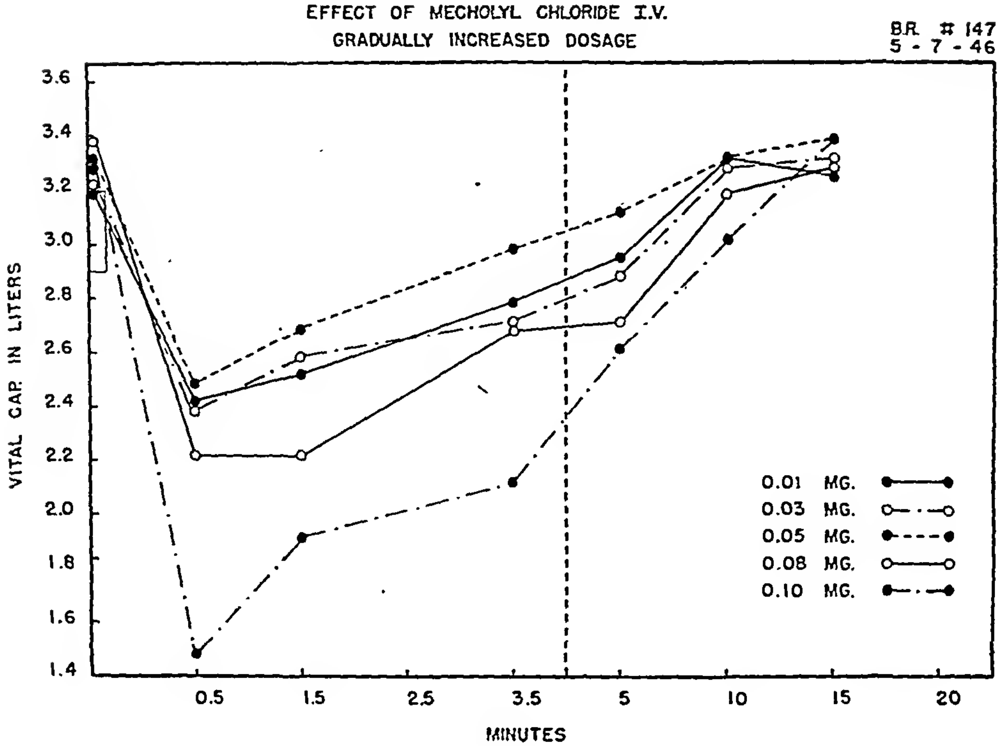

FIG. 2

SUMMARY

The reaction of the tracheobronchial tree, measured chiefly by changes in the vital capacity, to mecholyl chloride administered intramuscularly in doses of 0.25 to 6.0 mgm. was determined in 10 normal subjects, 11 patients with uncomplicated hay fever, and 27 patients with active bronchial asthma or a history of typical asthmatic attacks in the past. In the latter 2 groups, the reaction was compared with that due to parenteral histamine administered chiefly by the intravenous route in doses of 0.02 to 0.04 mgm. of the base. In the asthmatic group the reaction to intramuscular mecholyl chloride was studied in detail. The reaction to mecholyl chloride given intravenously and by nebulization was also studied in a few cases.

In the normal group, 3 subjects had a slight reduction in vital capacity after the intramuscular injection of 6 mgm. mecholyl chloride though all noted a feeling of tightness in the chest. In the group of 11 patients with hay fever, all had a slight reduction in vital capacity following the administration of 6 mgm. mecholyl chloride intramuscularly and in 7 cases the reduction was 5 per cent or greater. By contrast, in this latter group the intravenous administration of 0.02 to 0.03 mgm. histamine base caused a reduction in vital capacity in *6 of the 11 subjects but* in only *2* cases was the decrease 5 per cent or greater.

In the group of *27* subjects with bronchial asthma or a history of typical asthmatic attacks in the past all had a reduction in vital capacity after the intramuscular injection of from 1 to 6 mgm. mecholyl and in only 1 instance was the decrease less than 5 per cent. On the other hand, following the administration of parenteral histamine 23 subjects had a reduction in vital capacity, and in 22 of these cases the reduction was 5 per cent or greater. It appeared that patients with hay fever and asthmatic subjects were more reactive to mecholyl chloride than to histamine and that both drugs acted by different mechanisms on the hyperresponsive respiratory tract of these individuals.

The pattern of reaction of the tracheobronchial tree to mecholyl chloride, as measured by the reduction in vital capacity, was similar in responsive individuals, though the degree of reaction varied from patient to patient and in the same patient with the degree of asthma present at the time the

tests were performed. With increasing doses of the drug, an increased reduction in vital capacity resulted.

The intravenous injection of 0.005 to 0.6 mgm. mecholyl chloride produced a more pronounced decrease in vital capacity than the intramuscular injection, with its most notable effect in about 30 seconds after the injection. Nebulization of a 1:40,000 solution of mecholyl chloride produced a pronounced reduction in vital capacity which suggested that the tracheobronchial tree may be more reactive to the drug administered by this route.

The degree of extrapulmonary reaction to mecholyl chloride as indicated by flushing, sweating, salivation, tachycardia, and a feeling of warmth was no more notable in the asthmatic group than in the hay fever or normal groups. Paroxysmal auricular fibrillation occurred in 1 patient following a dose of 6 mgm. of mecholyl chloride intramuscularly.

BIBLIOGRAPHY

1. Curry, J. J., The action of histamine on the respiratory tract in normal and asthmatic subjects. J. Clin. Invest., 1946, 25, 785.
2. Barker, L. F., and Sladen, F. J., Tr. Assn. Am. Phys., 1912, 27, 471.
3. Alexander, H. L., and Paddock, R., Bronchial asthma: response to pilocarpin and epinephrin. Arch. Int. Med., 1921, 27, 184.
4. Starr, I., Jr., Elsom, K. A., Reisinger, J. A., and Richards, A. N., Acetyl-beta-methyl cholin. I. The action on normal persons: with a note on the action of the ethyl ether of beta-methyl cholin. Am. J. M. Sc., 1933, 186, 313.
5. Starr, I., Jr., Acetyl-beta-methyl cholin. III. Its action on paroxysmal tachycardia and peripheral vascular disease with a discussion of its action in other conditions. Am. J. M. Sc., 1933, 186, 330.
6. Villaret, M., Vallery-Radot, P., Justin-Besancon, L., and Claude, F., Preliminary research: On the attacks produced in certain asthmatic subjects by certain choline esters. Compt. Rend. de la Soc. Biol. 1934, 116, 1343.
7. Moll, H. H., The action of parasympathetic-mimetic drugs in asthma. Quart. J. Med., 1940, 9, 229.
8. Dautrebande, L., and Philippot, E., Experimental attacks of asthma by aerosols of carbamino choline in man and treatment by aerosol of phenylamino propane. Presse. Méd., 1941, 49, 942.
9. Ellis, L. B., and Weiss, S., Study of cardiovascular responses in man to intravenous and intra-arterial injection of acetyl choline. J. Pharmacol. & Exper. Therap., 1932, 44, 235.
10. Carmichael, E. A., and Fraser, F. R., The effects of acetyl choline in man. Heart, 1933, 16, 263.
11. Hurtado, A., and Kaltreider, N. L., Studies of total pulmonary capacity and its subdivisions. VII. Observations during the acute respiratory distress of bronchial asthma and following the administration of epinephrine. J. Clin. Invest., 1934, 13, 1053.
12. Eppinger, H., and Hess, L., Vagotonia, A Clinical Study in Vegetative Neurology, 2nd ed., New York, Nervous & Mental Division Publishing Co., 1917.
13. Milhorat, A. T., The choline esterase activity of the blood serum in disease. J. Clin. Invest., 1938, 17, 649.
14. Brown, E. A., Nobili, C., Sannella, T., and Wadsworth, G. P., Dyspnea and diminished vital capacity as a symptom and a sign in hay fever. Dis. of Chest, 1946, 12, 205.
15. Curry, J. J., The effect of antihistamine substances and other drugs on histamine bronchoconstriction in asthmatic subjects. J. Clin. Invest., 1946, 25, 792.

THE EFFECT OF ORALLY AND INTRAVENOUSLY ADMINISTERED AMINO ACID MIXTURES ON VOLUNTARY FOOD CONSUMPTION IN NORMAL MEN

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Intravenous and oral protein alimentation using hydrolysates of casein for treatment and prevention of protein deficiency states is receiving increasing recognition. Emphasis has been centered around nitrogen metabolism in such surgical conditions as shock (1), burns (2), blood loss (3, 4), wound healing (5, 6, 7), convalescence (8, 9), and postoperative infections (10, 11). Numerous investigations (12 to 15) have shown that positive nitrogen balance can be maintained for short periods of time in patients receiving intravenous amino acid mixtures as the only source of protein. Another of the major indications for parenteral nitrogen feedings is in patients with debilitating diseases who are unable or unwilling to take adequate food by mouth. In such cases the aim is to supplement the oral nitrogen intake by the use of intravenous protein hydrolysates to provide for the restoration of depleted protein reserves and the daily protein requirements.

During the course of previous investigations in this hospital it was observed that individuals who received intravenous injections of an acid hydrolysate of casein ("Parenamine") failed to eat the normal amount of food at the subsequent meal and many complained of loss of appetite. Reports of the occurrence of nausea, vomiting and anorexia associated with the administration of protein hydrolysates are common (12, 16–23). The symptoms of intolerance to these preparations occur most frequently upon rapid injection and constitute one of the disadvantages to their practical use.

Since nausea, vomiting, and anorexia following the infusion of protein digests might result in a decrease in food intake, the advantages offered by intravenous alimentation could thus be offset by a decrease in the consumption of food. The present investigation was undertaken to determine to what extent the administration of solutions of amino acid mixtures would influence the voluntary food intake in normal individuals. Studies were also carried out on the relationship of the rate of injection of amino acid mixtures to the occurrence of anorexia, nausea, and vomiting.

EXPERIMENTAL

Eight normal healthy adult males and 1 patient with portal cirrhosis were chosen for this study. They were permitted to be ambulatory except for the time required to complete the infusions. Each subject was offered a standard diet which supplied 3,544 calories. It contained approximately 135 grams of protein, 180 grams fat and 347 grams carbohydrate. Each item of uneaten food was weighed and the amount consumed was calculated as the difference between the weight of food offered and the weight of that returned. The items in the diet were varied to permit some choice. From the weight of the food consumed it was possible to calculate by the use of standard reference tables (24) the total calories and the amount of fat, carbohydrate, and protein consumed each day.

The general plan of the study was to keep the subject on the diet for a few days, then give amino acid preparations either orally or intravenously along with the diet, and note any effect on the food consumption. The days during which added protein was given will be referred to in this report as the *experimental period;* it lasted from 3 to 7 successive days when the supplemental protein was given orally and from 3 to 6 successive days, usually 3, when it was given intravenously. The subjects were under continuous observation for from 21 to 35 days. Each individual was studied for at least 2 experimental periods each of which was preceded and followed by control periods of not less than 3 days. Three amino acid preparations were used and they will be designated mixture I, II, and III.[1]

[1] Mixture I is an enzymic digest of casein and pork pancreas and is marketed under the trade name "Amigen," Mead Johnson and Co. Mixture II is an acid hydrolysate of casein to which tryptophane is added. It is marketed as "Parenamine" and was supplied through the courtesy of Frederick Stearns and Co. Mixture III is a synthetic amino acid mixture containing the ten essential amino acids to which glycine is added. The amounts used were suggested by Madden, S. C., and Clay, W. A. (J. Exper. Med., 1945, 82, 65) and referred to by them as mixture "VUJ." It was supplied through the courtesy of Merck and Co.

The sequence of amino acid mixtures used during the successive experimental periods was varied, as shown in Figures 1A, 1B and 2. Two subjects received only one of the mixtures, 7 subjects received 2 mixtures, and 2 of the subjects also received a 10 per cent glucose solution and physiological saline solution interspersed with the solutions of amino acid mixtures in order to obtain information on the effect that these solutions had on the food consumption (Figure 1B). In 4 subjects the effect of using casein hydrolysates by the oral route was investigated for 5 experimental periods and in these studies only mixture I was used.

The amount of mixtures given on each experimental day was equivalent to 100 grams of protein.[2] The volume of the solution administered daily was 1,440 ml. given in three 480-ml. infusions following each meal. Care was taken to arrange infusions so that they would not interfere with meals. The final concentration of amino acid mixture I and II was 8.3 per cent and of mixture III, 8 per cent. The speed of the infusions was purposely varied so that the 480 ml. of fluid was delivered in from 60 to 180 minutes. The rate of protein administration, therefore, varied from 13 grams to 40 grams per hour (Table II).

RESULTS

The results of the influence of oral and intravenous protein supplemental feedings on the amount of food voluntarily consumed during all of the control and experimental periods are summarized on Table I and Figures 1A, 1B, and 2. It can be seen that during the control period when the only source of food was the basal diet, the daily caloric intake remained relatively constant in all subjects. It is also evident that when amino acid Mixture I was given by mouth as the supplemental feeding (Figure 1A) there was little effect on the amount of food eaten. These changes, expressed as calories consumed per day, are interpreted as not being significant. Five subjects received amino acid Mixture I parenterally, and, of these, 4 had little or no depression of the caloric intake. The one patient in whom a depression of appetite occurred (Case No. 2, Figure 1A), had an intake of 67 per cent of the basic control diet. In sharp contrast are the results which occurred in all 7 subjects who received amino acid Mixture II by the intravenous route. There was a constant reduction in the food voluntarily consumed; the average fall was to 61 per cent of the normal consumption with variations ranging from 37.8 per cent to 87 per cent (Table II). This reduction occurred in each of 2 experimental periods in 1 subject (Patient 9, Figure 2) when he was given this preparation. The same results followed the giving of this solution irrespective of whether its use preceded or followed the other 2 mixtures.

Amino acid Mixture III was administered to 3 men (Patients 6, 7, and 8, Figure 2) and no measurable change of food consumption occurred during or following the preparation. Ten per cent glucose and physiological saline infusions were

TABLE I

The effect of amino acid preparations on voluntary food consumption

Patient no.	Initials	Period of study	Days on study	Average total caloric intake, cal. per day	Food consumption, per cent of control
1	G. S.	Control	16	3,227	
		Preparation I orally	9	3,313	103.0
2	S. T.	Control	34	2,257	
		Preparation I orally	7	2,455	104.5
		Preparation I I.V.	10	1,523	67.6
		Preparation II I.V.	3	853	37.8
		Saline I.V.	3	1,948	86.3
		10 per cent Glucose I.V.	3	2,239	99.5
3	H. W.	Control	17	2,783	
		Preparation I orally	5	2,612	93.6
		Preparation I I.V.	3	2,461	88.3
		Preparation II I.V.	3	1,693	60.8
4	J. B.	Control	22	1,807	
		Preparation I orally	5	1,805	99.9
		Preparation I I.V.	3	1,629	90.0
		Preparation II I.V.	3	793	43.0
5	W. M.	Control	24	2,489	
		Preparation I I.V.	6	2,249	90.7
		Preparation II I.V.	4	1,493	60.2
		Saline I.V.	3	2,285	91.8
		10 per cent Glucose I.V.	3	2,829	111.7
6	E. G.	Control	18	2,073	
		Preparation II I.V.	4	1,600	77.2
		Preparation II I.V.	4	1,241	59.9
7	F. S.	Control	13	3,061	
		Preparation II I.V.	3	1,907	62.4
		Preparation III I.V.	3	3,089	101.0
8	G. E. F.	Control	18	2,402	
		Preparation III I.V.	3	2,706	112.2
		Preparation II I.V.	3	2,081	87.0
9	G. C.	Control	23	3,211	
		Preparation I I.V.	3	3,205	99.8
		Preparation III I.V.	3	2,711	85.0

[2] The total nitrogen of each amino acid mixture, based on the data supplied by the manufacturer, was multiplied by 6.25 to give the equivalent of the number of grams of protein.

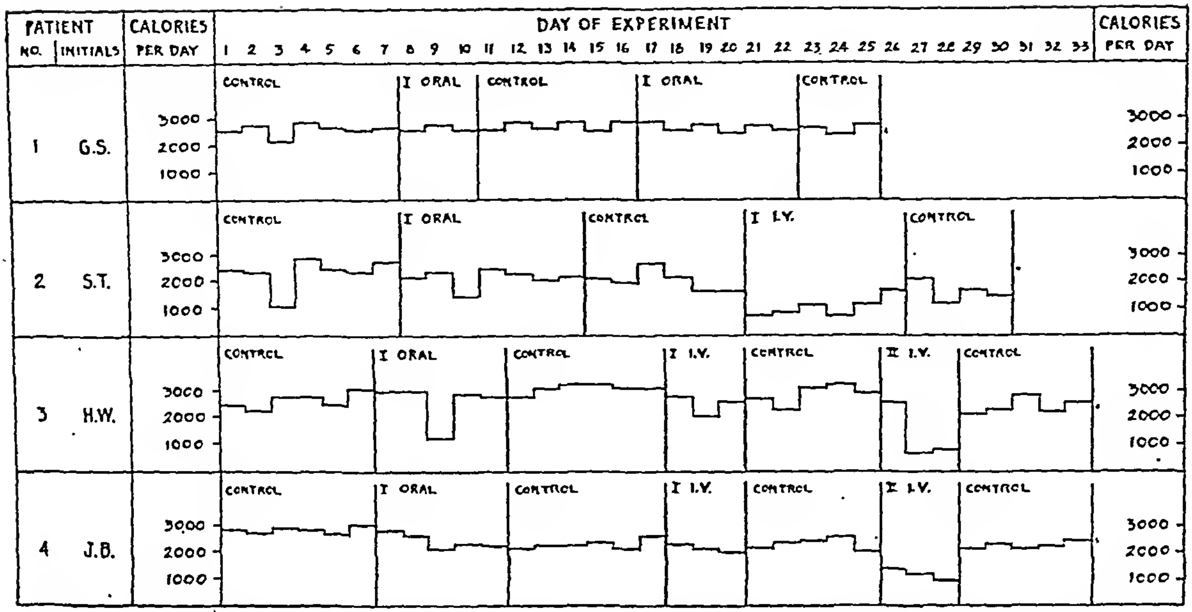

FIG. 1A. EFFECT OF AMINO ACID MIXTURE I ADMINISTERED ORALLY AND INTRAVENOUSLY AND AMINO ACID MIXTURE II ADMINISTERED INTRAVENOUSLY UPON THE VOLUNTARY FOOD INTAKE

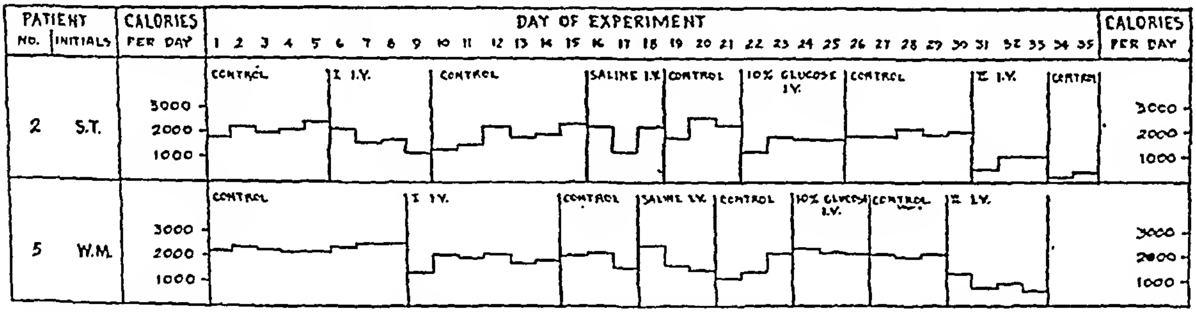

FIG. 1B. EFFECT OF THE INTRAVENOUS ADMINISTRATION OF AMINO ACID MIXTURES I AND II UPON THE VOLUNTARY FOOD INTAKE, COMPARED WITH THE INTRAVENOUS ADMINISTRATION OF PHYSIOLOGICAL SALINE AND TEN PER CENT GLUCOSE SOLUTION

given to 2 individuals and the results are shown in Figure 1B. These 2 commonly employed solutions produced little effect upon the appetite of these men.

In many experimental periods, notably with patients Nos. 2, 4, 5, 6, and 7, there occurred a stepwise depression of food consumption with each additional day during the infusion of supplementary protein. In some instances this progressive depression extended into the first or even the second day of the following control period. This tendency toward a progressive loss of appetite on the last days of the experimental periods and a lag or overlap into the subsequent control period was particularly notable with the use of Mixture II; it occurred to a lesser degree with Mixture I, and was not observed with Mixture III.

It was observed that an important factor which influenced the consumption of food in these subjects was the reactions occurring with or soon after the subject received the amino acid mixture. In the case of amino acid Mixture III no reactions occurred, but Mixture II produced nausea and vomiting in many of the subjects. Another important complaint of the persons receiving the latter preparation was a burning sensation in the arm while receiving the mixture. The burning extended up the arm along the vein to the axilla and was occasionally followed by thrombophlebitis. The thrombosis and pain in the arms made pro-

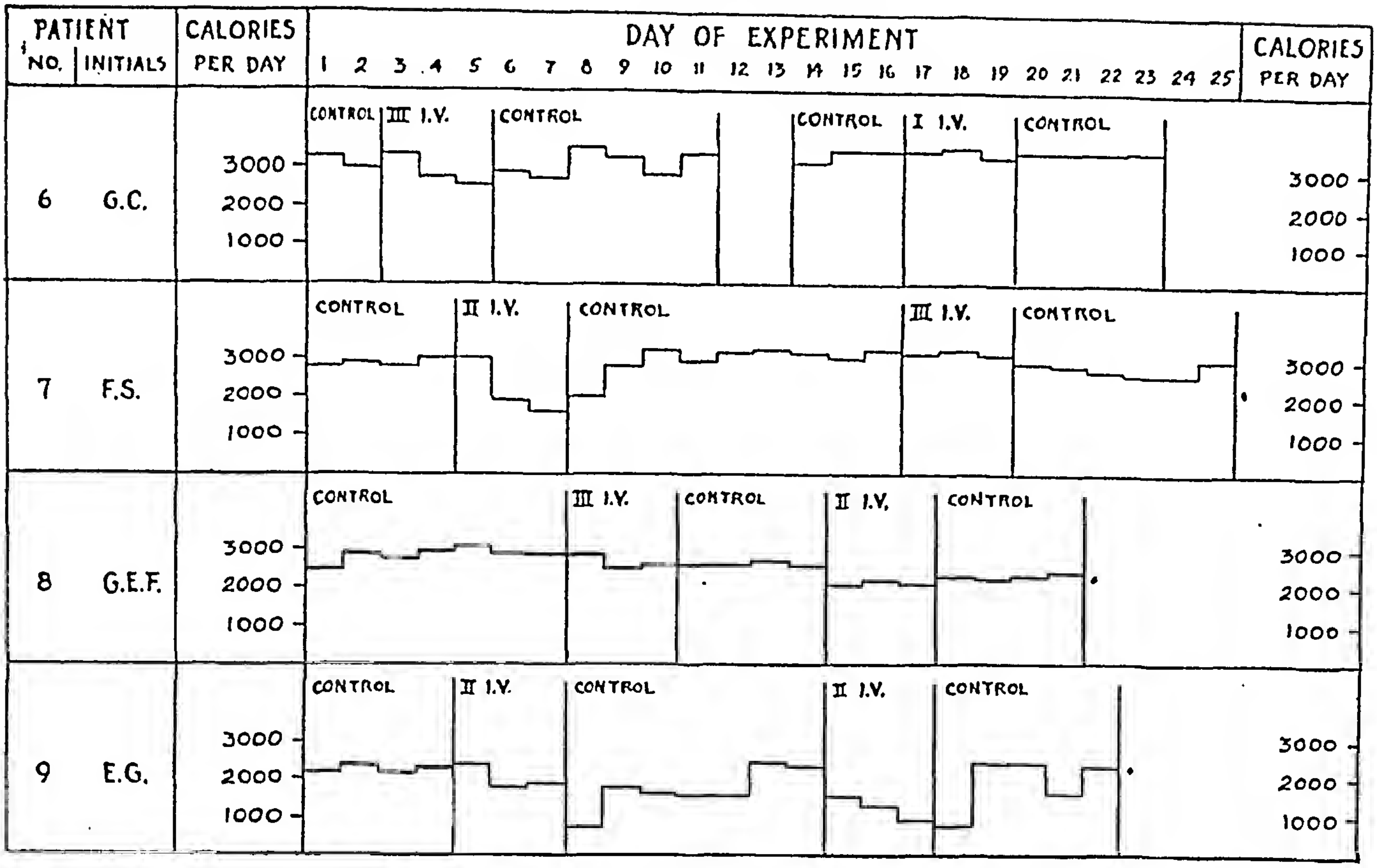

FIG. 2. EFFECT OF INTRAVENOUS ADMINISTRATION OF AMINO ACID MIXTURES I, II, AND III UPON THE VOLUNTARY FOOD INTAKE

longed administration of this mixture difficult. The depression of appetite as reflected in the decreased food intake was not directly related to the presence or absence of the venous thrombosis because the depression occurred in subjects in whom no vein lesions developed, but the associated pain probably contributed to the poor appetite to some extent. Some of the men complained that after receiving any of these preparations they had an unpleasant taste that was similar to the smell of the casein digest. This peculiar taste was reported by a number of patients and remained for a few days after the administration of the amino acids stopped.

TABLE II

Effects of rates of intravenous administration of amino preparation on voluntary food consumption

Patient no.	Initials	Amino acid mixture I				Amino acid mixture II				Amino acid mixture III			
		Rate protein		Change in food intake, per cent of control	Nausea or vomiting	Rate protein		Change in food intake, per cent of control	Nausea or vomiting	Rate protein		Change in food intake, per cent of control	Nausea or vomiting
		grams per hr.	*ml. per min.*			*grams per hr.*	*ml. per min.*			*grams per hr.*	*ml. per min.*		
2	S. T.	13	3.2	−23	N	15	3.7	−63	V				
3	H. W.	40	10.0	−10	*	26	6.5	−37	V				
4	J. B.	22	5.5	−10	*	21	5.3	−57	*				
5	W. M.	24	6.2	−12	*	24	6.2	−39	V				
6	E. G.					30	7.6	−23	*				
						14	3.5	−40	V				
7	F. S.					18	4.5	−38	V	22	5.5	0	0
8	G. E. F.					27	6.7	−13	V	40	10.0	+12	0
9	G. C.	32	8.0	0	0					26	6.5	−15	0

* Information incomplete.

The results of the studies of the relationship of rate of infusion to the food consumption during intravenous protein feedings are summarized in Table II. Some of the peculiarities involved in the rate of administration of these products can be seen in the data derived from patient H. W. (No. 3). Preparation I was given at an average rate equivalent to 40 grams per hour with no ill effects, while Preparation II caused a marked depression in food consumption when given at an average rate of 26 grams of protein per hour. Patient J. B. (No. 4) received both Mixtures I and II at almost identical rates. In this case the drop in calories consumed per day occurred only with the latter mixture. An unusual effect is presented by patient E. G. (No. 9) who received Mixture II on 2 different experimental periods of 3 days each. The rate was 30 grams per hour during the first period and only slight step-wise depression of the total food consumed resulted. At the second experimental period the rate of infusion was approximately ½ that of the first period (14.5 grams per hour) and on this occasion this same mixture caused a pronounced fall in the amount of food eaten. Mixture III did not cause any change in the appetite as judged by the amount of food consumed, and the rate of its infusion was comparable to or faster than those used with the other mixtures.

In these cases where there was some reduction in the voluntary intake after the giving of amino acid digests, the question of selective reduction of any one of the major foodstuffs was investigated. The amount of fat, carbohydrate, and protein eaten by each subject was calculated and expressed as the ratio of Protein:Fat:Carbohydrate. Table III is a summary of some of these ratios. There appears to be no constant decrease in consumption of any given class of foodstuffs after amino acid therapy. Variations in the ratios appear, but no trend is evident. In other words, when these individuals cut down on eating they did not tend to avoid selectively the high protein foods and to eat only foods high in carbohydrates or fats.

DISCUSSION

Co Tui (9) has pointed out that one of the major drawbacks to the clinical use of casein hydrolysates for intravenous protein feedings is the difficulty in giving enough to supply the nitrogen and caloric needs. This is particularly true if it is desired to give large amounts of protein building materials. Elman (25), in presenting a régime for intravenous alimentation of patients suf-

TABLE III

Effect of intravenous amino acid therapy on the ratio of protein: fat: carbohydrate voluntarily consumed
Ratio of protein: fat: carbohydrate of standard diet offered 1:1.32:2.56

Pt. no.	1	2	3	4	5	6	7	8	9
Pt. initials	G. S.	S. T.	H. W.	J. B.	W. M.	E. G.	F. S.	G. E. F.	G. C.
	Fat:CHO*	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO	Fat:CHO
Control	1.63:2.55	1.38:2.50	1.77:3.00	1.36:3.06	1.34:1.94				
Oral amino acid I	1.39:3.03	1.20:2.45	1.54:2.89	1.42:3.22	1.43:1.66				
Control	1.44:2.60	1.25:2.58	1.54:2.73	1.47:3.22	1.61:1.68				
Oral amino acid I	1.49:2.66								
Control	1.51:2.99								
I.V. amino acid I		1.32:2.66	1.56:3.13	1.57:3.90					
Control		1.37:2.50	1.61:2.98	1.47:3.22					
I.V. amino acid I		1.77:3.70							
Control		1.33:2.49							
I.V. phys. saline		1.28:2.28			1.30:1.70				
Control		1.35:2.42			1.34:1.79				
I.V. 10 per cent glucose		1.52:2.95			1.64:1.83				
Control		1.56:3.20			1.40:1.68	1.47:3.63			
I.V. amino acid II		1.52:1.76	1.92:3.55	1.84:5.35	2.60:2.54	1.51:3.75			
Control				1.55:3.50		1.80:4.00	1.16:2.80	1.39:3.17	1.13:2.43
I.V. amino acid III							1.13:2.40	1.38:3.03	1.15:2.05

* Figures in the table are fat: carbohydrate ratio, protein = 1.

fering from severe protein deficiency, advised the giving of both amino acids and glucose. The sum of the volumes of solutions given per 24-hour period amounted to 3,000 ml., and the total caloric intake was only 1,200 calories, an amount which usually would not be sufficient for a normal subject to maintain his weight. In this investigation the daily dose of the equivalent of 100 grams of protein was arbitrarily chosen because it was considered an amount adequate to provide the generally recommended allowance of 1 gram of protein per kilogram of body weight and a margin to replace depleted protein reserves.

A practical consideration in giving intravenous protein supplements is to employ a method that will not interfere with the patient's daily food intake. In this study 3 injections were given daily, and the individual infusions were completed in not more than 3 hours. Longer infusions were considered inadvisable from the standpoint of comfort to the patient and because the presence of the needle in the vein was a factor which mechanically interfered with the eating of the regular meals. The rates used with Mixture I and II exceeded those usually recommended. Mixture III was frequently given at rates twice those used with the casein digests. The data regarding the rate of infusion in the present study do not permit any final conclusions.

One of the requirements for an acceptable intravenous preparation is that it must be injectable in adequate amounts without producing nausea, vomiting, or other unpleasant reactions. The relationship of reactions which occurred during or following the infusions to the depression of the appetite is a matter of great practical importance. The one constant finding was that, of the 3 mixtures used, the greatest impairment in the appetite occurred during those experimental periods when Mixture II was given. However, it is noteworthy that none of the subjects given Mixture III had any side-effects, and in this same group no significant reduction in the consumption of food was observed. When these same patients received Mixture II impairment of the appetite occurred.

At the present time there is no satisfactory explanation for the occurrence of nausea and vomiting. Albanese (26) questioned if these toxic effects were due to unnatural isomers of certain amino acids which cannot be utilized by the human body. Hopps (27) suggests that the presence of some histamine-like substances, peptones or tyramine as agents may explain the frequently observed reactions of flushing, sensation of warmth, and nausea; but he gives no experimental data to support these ideas. Madden and associates (22) have shown that both glutamic and aspartic acids when added to a mixture of pure amino acids will cause violent vomiting in dogs. The effect of these 2 amino acids upon the voluntary food consumption and as a factor causing emesis in humans is being investigated and will be reported in a separate communication. Hoffman, Kozoll, and Osgood (28) have presented evidence that the height of the amino acid nitrogen content of the blood is probably the factor responsible for producing nausea. They found that if the rate is sufficiently rapid to get a blood amino acid nitrogen value of 10 mgm. per cent or greater, nausea will result. These studies were carried out using Mixture II. Hecht (21), using Mixture II, has suggested that perhaps the rapidity of the rise of the amino acid blood levels and not the actual levels themselves were responsible for the side effects.

The progressive step-wise reduction in the amount of food voluntarily consumed during the succeeding days of the experimental periods occurred with such regularity, particularly with Mixture II, that it must be considered more than a chance occurrence. The overlap into the subsequent first and second days of the control period and the gradual recovery were the usual findings in these cases. At present there is no explanation for this occurrence.

These studies raise the question as to whether the advantages of giving large doses of amino acid mixtures intravenously are offset by increased urinary nitrogen "wastage" which would be expected to accompany the rapid rates of infusion. In the search for a more acceptable preparation for intravenous use, this important point will require investigation.

SUMMARY

The effect of the oral and intravenous administration of mixtures of amino acids on the volun-

tary food consumption in normal human subjects was studied. Three preparations were used: an enzymic hydrolysate of casein (I), an acid hydrolysate of casein (II), and a mixture of the 10 essential amino acids plus glycine (III). The enzymic digest had little effect on the appetite whether it was given orally or intravenously. The acid hydrolysate of casein consistently produced a marked depression in the voluntary food consumption during and following its intravenous administration. The mixture of the essential amino acids had no depressing effect on the appetite. This product was the best tolerated of the 3 tested and could be given at exceedingly rapid rates without any ill effects. When there was reduction in the amount of food eaten, it consisted of a general lack of interest in food rather than a selective rejection of a certain class of foodstuff.

BIBLIOGRAPHY

1. Elman, R., Acute protein deficiency (Hypoproteinemia) in surgical shock due to severe hemorrhage and in burns, intestinal obstruction and general peritonitis, with special reference to use of plasma and hydrolized protein. J. A. M. A., 1942, 120, 1176.
2. Co Tui, Wright, A. M., Mulholland, J. H., Barcham, I., and Breed, E. S., Nutritional care of cases of extensive burns; with special reference to oral use of amino-acids (Amigen) in 3 cases. Ann. Surg., 1944, 119, 815.
3. Elman, R., and Lischer, C. E., Amino-acids, serum and plasma in replacement therapy of fatal shock due to repeated hemorrhage; experimental study. Ann. Surg., 1943, 118, 225.
4. Nicholl, R. J., Boucher, W. F., and Prince, R. W., Hemorrhagic shock; relative effect of amino acids, Amigen and gelatin in dogs. Surg. Gynec. & Obst., 1945, 80, 181.
5. Co Tui, Value of protein and its chemical components (amino acids) in surgical repair. Bull. New York Acad. Med., 1945, 21, 631.
6. Thompson, W. D., Ravdin, I. S., and Frank, I. L., Effect of hypoproteinemia on wound disruption. Arch. Surg. 1938, 36, 500.
7. Howard, J. E., Winternitz, J., Parson, W., Bigham, R. S., and Eisenberg, H., Studies on fracture convalescence; influence of diet on post-traumatic nitrogen deficit exhibited by fracture patients. Bull. Johns Hopkins Hosp., 1944, 75, 209.
8. Howard, J. E., Protein metabolism during convalescence after trauma; recent studies. Arch. Surg., 1945, 50, 166.
9. Co Tui, Some clinical aspects of protein nutrition. J. Amer. Dietet. A., 1946, 22, 97.
10. Cannon, P. R., Protein metabolism and resistance to infection. J. Michigan M. Soc., 1944, 43, 323.
11. Cannon, P. R., Importance of proteins in resistance to infection. J. A. M. A., 1945, 128, 360.
12. Madden, S. C., Bassett, S. H., Remington, J. H., Martin, F. J. C., Woods, R. R., and Shull, F. W., Amino acids in therapy of disease; parenteral and oral administrations compared. Surg. Gynec. & Obst., 1946, 82, 131.
13. Bassett, S. H., Woods, R. R., Shull, F. W., and Madden, S. C., Parenterally administered amino acids as source of protein in man. New England J. Med., 1944, 230, 106.
14. Elman, R., Charnas, R., and Davey, H. W., Ceiling of utilization of nitrogen. Arch. Surg., 1943, 47, 216.
15. Brunschwig, A., and Corbin, N., Clinical study of relative efficiency for nitrogen metabolism of casein administered intravenously and protein ingested by mouth. Surgery, 1943, 14, 898.
16. Davis, H. H., Amino acids intravenously in surgical patients. Nebraska M. J., 1945, 30, 51.
17. Hartmann, A. F., Parenteral administration of amino acids. J. Pediat., 1945, 26, 193.
18. Elman, R., Parenteral fluids and food in gastrointestinal disease. Bull. New York Acad. Med., 1944, 20, 220.
19. Brunschwig, A., Clark, D. E., and Corbin, N., Intravenous injection of casein digest (amino acids) in maintenance of nutrition; consideration of medico-military aspects. Mil. Surgeon, 1943, 92, 413.
20. Landesman, R., and Weinstein, V. A., Intravenous use of amino acids for nutritional purposes in surgical patient. Surg. Gynec. & Obst., 1942, 75, 300.
21. Hecht, H. H., Reactions to intravenously administered amino acids (casein hydrolysates). Am. J. M. Sc., 1946, 212, 35.
22. Madden, S. C., Woods, R. R., Shull, F. W., Remington, J. H., and Whipple, G. H., Tolerance to amino acid mixtures and casein digests given intravenously; glutamic acid responsible for reactions. J. Exper. Med., 1945, 81, 439.
23. Unna, K., and Howe, E. E., Toxic effects of glutamic and aspartic acid. Fed. Proc. 1945, 4, 138.
24. Bowes, A. P., and Church, C. F., Food values of portions commonly used. Philadelphia Child Health Society, Philadelphia, Penn. 1942.
25. Elman, R., The practical use of amino acids in protein nutrition. J. A. M. A., 1945, 128, 659.
26. Albanese, A. A., The utilization of d-amino acids by man; tryptophane, methionine and phenylalanine. Bull. Johns Hopkins Hosp., 1944, 75, 175.
27. Hopps, H. C., and Campbell, J. A., Immunologic and toxic properties of casein digest is prepared for parenteral administration. J. Lab. & Clin. Med., 1943, 28, 1203.
28. Hoffman, W. S., Kozoll, D. D., and Osgood, B., Blood chemical changes following intravenous administration of a casein hydrolysate to human subjects. Proc. Soc. Exper. Biol. & Med., 1946, 61, 137.

MINIMUM DOSAGE OF THIOUREA, GIVEN TOGETHER WITH IODINE MEDICATION, NECESSARY FOR THE PRODUCTION AND MAINTENANCE OF A REMISSION IN HYPERTHYROIDISM [1]

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It has been shown that thiourea in amounts smaller than those recommended for thiouracil can produce a satisfactory remission in hyperthyroidism (1). The thyroid-depressant effect is enhanced if thiourea is used in combination with strong solution of iodine (2, 3, 4). The administration of these two substances together results in a more rapid and more profound drop in the concentration of serum precipitable iodine than that observed with thiourea alone. Symptoms and signs of thyroid overactivity disappear along with or soon after the fall in serum precipitable iodine. Continued administration of thiourea, however, in the dosage initially used to produce a remission (280 to 210 mgm. daily) ultimately results in hypothyroidism in a number of patients. Ingestion of desiccated thyroid is followed by disappearance of the hypothyroidism and the serum precipitable iodine concentration returns to normal.

These facts suggest that the dosage initially employed was unnecessarily large. The present study arose from an attempt to avoid hypothyroidism by the use of the minimum effective dose of thiourea. To determine the magnitude of this, the effects of small doses of thiourea, 0.075 to 0.015 gram daily, together with iodine solution in inducing and maintaining a remission in hyperthyroidism have been studied. Results obtained with iodine medication alone, and those with amounts of thiourea greater than 0.075 gram are included for purposes of comparison.

MATERIALS AND METHODS

Data are presented on 4 groups of hyperthyroid subjects, some with diffuse goiter and some with adenomata. With but few exceptions the patients were not hospitalized. The diagnosis of thyroid overactivity was based primarily upon the presence of a concentration of serum precipitable iodine of 8.0 gamma per cent or more (5, 6). This was usually but not invariably accompanied by an increase in the basal metabolic rate above plus 20 per cent. In all patients the clinical history and findings were compatible with the diagnosis of hyperthyroidism. No toxic reactions were observed in any of the patients during treatment with thiourea.

RESULTS

A. Production of a remission in hyperthyroidism. The effects of 2 treatment régimes on the course of active hyperthyroidism, measured by changes in the serum precipitable iodine, have been studied in 89 patients.

(1) TREATMENT WITH IODINE AND THIOUREA. Fifty hyperthyroid patients were treated with thiourea together with strong solution of iodine. Of these patients, 9 received 0.280 gram of thiourea daily in a single dose; 12 were treated with a total of 0.210 gram daily taken in 3 equal doses 8 hours apart; 17 other patients were started on either 0.070 gram in a single dose, or on 0.075 gram divided into 3 equal doses of 0.025 gram each; the remaining 12 patients took 0.005 gram of thiourea 3 times daily for a total daily dose of 0.015 gram (Figure 1A, B, C, and D). Each patient in this group of 50 also received 5 drops of strong solution of iodine 3 times daily.

It is evident from the figure that equally satisfactory results were obtained with either 0.280 gram or 0.210 gram of thiourea daily in addition to iodine solution. At the end of 10 to 12 weeks of treatment or less, all but one patient in each of these 2 groups had developed concentrations of serum precipitable iodine of 8.0 gamma per cent or less (Figure 1A and B). The use of a smaller amount of thiourea, 0.07 gram daily, proved to be somewhat less effective. At the end of a similar period of treatment, 10 out of 13 patients had responded (Figure 1C). A daily intake of only 0.015 gram of thiourea, on the other hand, failed

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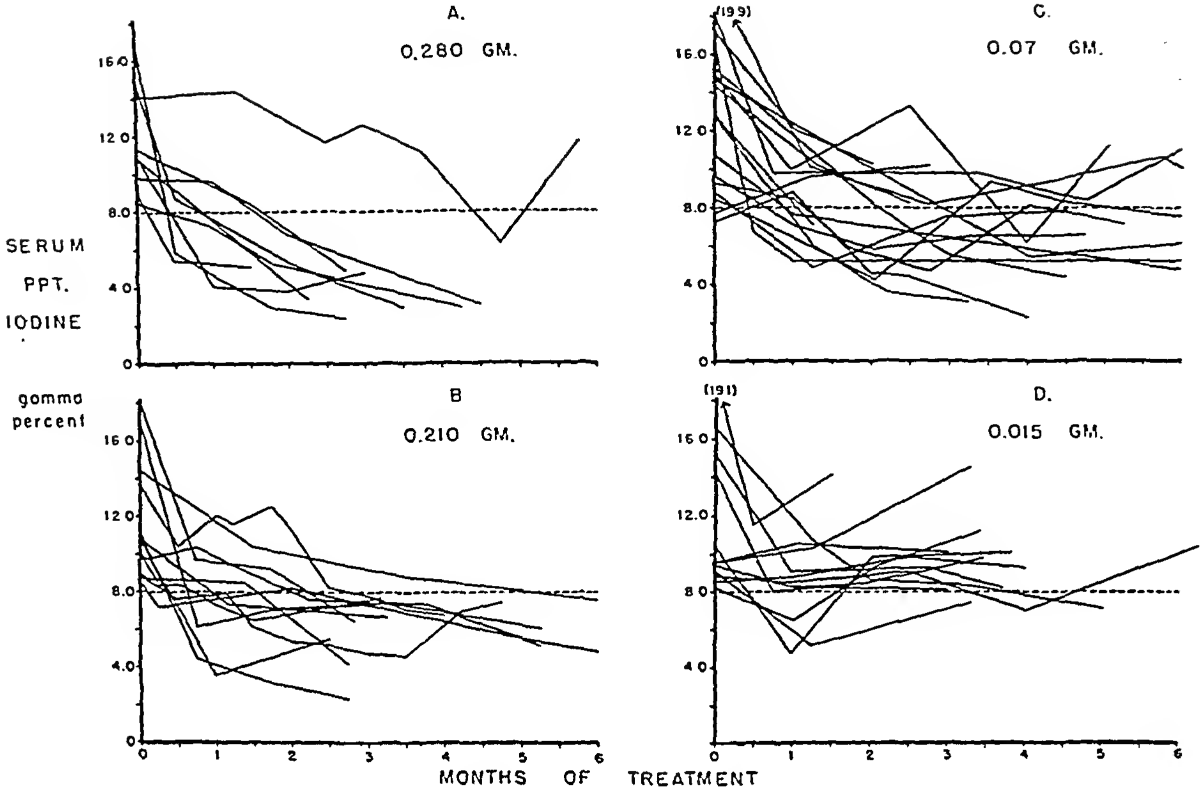

FIG. 1. TREATMENT OF HYPERTHYROIDISM WITH IODINE SOLUTION TOGETHER WITH THIOUREA

The return of the serum precipitable iodine to normal concentrations together with amelioration of the hyperthyroidism occurred almost as often in patients maintained on 0.07 gram of thiourea daily as it did in those on 0.280 or 0.210 gram. With doses as low as 0.015 gram daily, however, no thiourea effect is apparent; the occasional response observed is to be attributed to the iodine solution.

with but 1 exception to bring and keep the hyperthyroidism under control (Figure 1D). Moreover, in contrast to the findings in the preceding 3 groups, hypothyroid levels of serum precipitable iodine (4.0 gamma per cent or lower) did not develop in any of these patients.

The response to these various doses of thiourea is also reflected in the values of the serum precipitable iodine at selected points during the course of treatment. The averaged values of the serum precipitable iodine, for example, were quite comparable prior to treatment in the 4 groups. They were 11.7 ± 2.6, 11.8 ± 2.9, 12.8 ± 3.8, and 11.7 ± 3.6 gamma per cent in groups A, B, C, and D respectively. After 10 to 12 weeks of treatment, the average value of the serum precipitable iodine in patients in group A had declined to 5.7 ± 2.9 gamma per cent. The decrease was progressively less marked in groups B and C receiving smaller amounts of thiourea, since the average value in the former during the 10- to 12-week period was 6.5 ± 2.0 gamma per cent, and in the latter 6.9 ± 2.3. In group D, however, the average level of precipitable iodine during a comparable period was 9.5 ± 1.5 gamma per cent. The serum precipitable iodine therefore declined significantly in groups A, B, and C but not in group D. The differences in extent of decline among the first 3 groups are probably not significant.

(2) TREATMENT WITH IODINE ALONE. Thirty-nine patients were treated with strong solution of iodine alone, in a total daily dose of 15 drops. To simplify, graphic presentation results obtained with this therapy have been subdivided into 4 categories based upon changes in the concentration of the serum precipitable iodine (Figure 2A, B, C, and D). The serum precipitable iodine declined to and remained at euthyroid levels, 4.0 to 8.0 gamma per cent, in only 9 of the 39 patients (Figure 2A). In 6 other patients the serum precipitable iodine

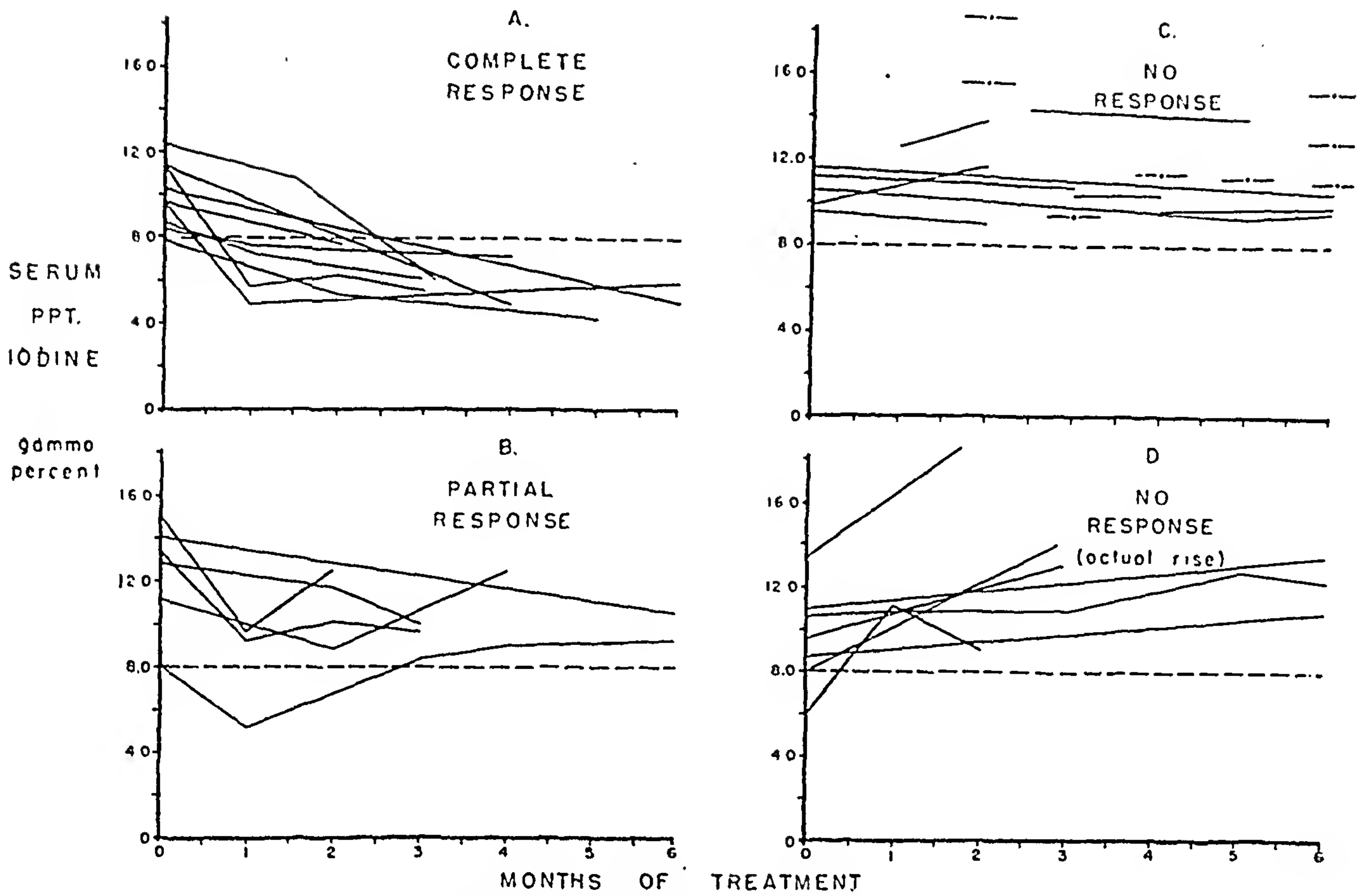

FIG. 2. PROLONGED TREATMENT OF HYPERTHYROIDISM WITH IODINE SOLUTION ONLY

Clinical improvement and euthyroid concentrations of serum precipitable iodine appeared and persisted in 9 of the 39 patients. In the remaining 30 patients the serum precipitable iodine, save for a temporary initial decrease in some instances, remained elevated and thyroid overactivity continued.

fell 2.0 gamma per cent or more, but in only 1 did the concentration become normal. This patient and several others showed a subsequent rise to the pretreatment levels (Figure 2B). In the majority of patients, 17 in number, the serum precipitable iodine either failed to change at least 2.0 gamma per cent or was still distinctly elevated despite prolonged treatment with iodine solution (Figure 2C). In the 7 remaining patients, the serum precipitable iodine actually increased during iodine treatment (Figure 2D).

In those few patients in whom the concentration of serum precipitable iodine fell to normal levels the hyperthyroidism largely disappeared. Some of the others also showed a measure of clinical improvement. This did not always correlate in either magnitude or direction with the change in the serum precipitable iodine. In the majority of patients, however, the clinical symptoms and signs of thyroid overactivity continued unabated or actually increased during treatment with the iodine solution. None of the patients developed hypothyroid concentrations of iodine.

B. Maintenance on reduced amounts of thiourea and on iodine solution. The effectiveness of 0.07, 0.05, 0.035, 0.025, and 0.015–0.010 gram doses of thiourea and 15 drops of iodine solution daily in maintaining the remission obtained by larger amounts of thiourea has been studied in 15 patients. Following the appearance of a stable euthyroid state the daily intake of the drug was reduced. In some of the patients the maintenance dose was decreased more than once. The strong solution of iodine, however, was continued throughout the entire treatment period in all 15 patients without alteration in dosage. Variations in the concentration of serum precipitable iodine following reduction in the dose of thiourea are presented in Table I together with other pertinent data.

The remission was maintained despite a reduction in the daily intake of thiourea to 0.07 gram

in 11 patients in whom the initial response had been induced by 0.280, 0.210, or 0.140 gram. Further decrease in the maintenance dose of thiourea to 0.050, 0.035, or 0.025 gram resulted in a recurrence of hyperthyroidism in only 1 out of 11 trials (Patient E. B.). Attempts to control thyroid overactivity with 0.015 to 0.010 gram of thiourea daily failed, however, in 5 out of 8 patients.

In the 4 patients in whom the serum precipitable iodine decreased to 4.0 gamma per cent or lower, the level was raised to euthyroid values in 2 (Patients F. W. and P. G.) and toward normal in the other 2 (Patients K. S. and S. L.) by reducing the daily intake of thiourea. Maintenance of a remission for prolonged periods of time is quite feasible, therefore, on 0.07 to 0.025 gram of thio-

TABLE I

Effects of reduction of dosage of thiourea following the initial remission of hyperthyroidism on thiourea and iodine solution

Patient	Dosage of thiourea	Duration from start of thiourea	Body weight	Pulse rate	Basal metabolic rate*	Serum precipitable iodine*
	grams	*weeks*	*kilograms*	*per minute*	*per cent*	*gamma per cent*
E. B.		0	73.2	100		8.3
	0.280	0–22	76.1	84		7.4
	0.070	22–28	76.0	84		8.0
	0.050	28–45	75.0	82		8.6
M. M.		0	65.5	70	+39	14.2
	0.140	0–27	75.6	56	−9	4.5
	0.050	27–55	75.8	60	+2	4.3
K. S.		0			+36	11.1
	0.210	0–10	69.3	72	−19	2.4
	0.070	10–13	69.7	70	−26	2.4
	0.035	13–21	60.0	64	−28	3.2
S. L.		0	49.9	84	+41	9.8
	0.280	0–52	58.2	76	−4	1.7
	0.070	52–69	62.5	76	−18	2.9
	0.025	69–75	61.4	70	−14	2.6
M. B.		0	62.7	88	+18	9.9
	0.210	0–21	60.9	52	−4	5.4
	0.075	21–28	60.6	52	−11	6.3
	0.025	28–45	60.7	56	−6	7.5
E. C.		0	57.6	72	+7	8.7
	0.210	0–11	55.4	60	+4	4.2
	0.070	11–25	58.6	54	−13	4.4
	0.035	25–39	59.6	62	−11	5.4
	0.025	39–67	54.4	56	−10	6.1
J. H.		0	58.0	114		14.1
	0.140	0–20	58.6	76	+12	8.0
	0.070	20–28	58.6	80	+11	7.3
	0.025	28–42	56.5	68	+6	7.4

TABLE I—*Continued*

Patient	Dosage of thiourea	Duration from start of thiourea	Body weight	Pulse rate	Basal metabolic rate*	Serum precipitable iodine*
	grams	*weeks*	*kilograms*	*per minute*	*per cent*	*gamma per cent*
S. D.		0	84.5	96	+66	13.3
	0.140	0–11				5.8
	0.070	11–30	83.2	64	+13	7.0
	0.015	30–38	84.0	66		6.2
E. H.		0	72.7	84	+40	15.2
	0.075	0–18	75.4	68	−1	4.4
	0.015	18–30	75.3	66	−1	6.5
H. S.		0	64.2	80	+14	9.3
	0.075	0–11	67.2	68	−5	4.9
	0.015	11–31	71.3	68	−9	7.7
F. W.		0	43.3	120		12.2
	0.210	0–24	45.0	82		2.7
	0.070	24–64	47.2	84		5.9
	0.015	64–76	47.0	88		9.2
G. W.		0	41.2	112	+48	17.0
	0.210	0–31	49.7	76	+6	6.1
	0.075	31–37	49.6	80		7.0
	0.015	37–43	46.4	100		11.6
P. G.		0	60.8	72	+42	22.2
	0.280	0–32	65.7	60	−23	2.9
	0.070	32–40	67.9	52	−25	3.9
	0.035	40–63	63.2	48	+1	6.9
	0.013	63–80	65.4	64	+8	13.1
M. A.		0	59.2	66	−14	9.7
	0.070	0–19	60.4	68	−7	6.4
	0.035	19–25	61.4	64	−5	7.4
	0.025	25–37	61.3	60	−10	5.2
	0.013	37–51	60.5	68	−13	9.4
I. A.		0	57.4	78	+29	12.8
	0.210	0–22	61.4	56	−20	7.4
	0.075	22–28	60.5	58	−16	6.3
	0.010	28–46	60.6	50	−10	9.9

* At end of period.

urea with only rare exacerbation of hyperthyroidism. Reductions to these amounts also prevent or correct myxedema. Amounts of thiourea as low as .015 gram, however, appear to be quite as ineffective in maintaining a remission as they were found to be in producing the initial response.

C. Effect of withdrawal of thiourea. In 10 patients, thiourea has been discontinued after administration in various amounts for 5 to 24 months, together with strong iodine solution. In 3 of these, the iodine solution was stopped along with the thiourea. In the remaining 7, it was continued as the sole medication. All 10 patients in this group were free of hyperthyroid symptoms and

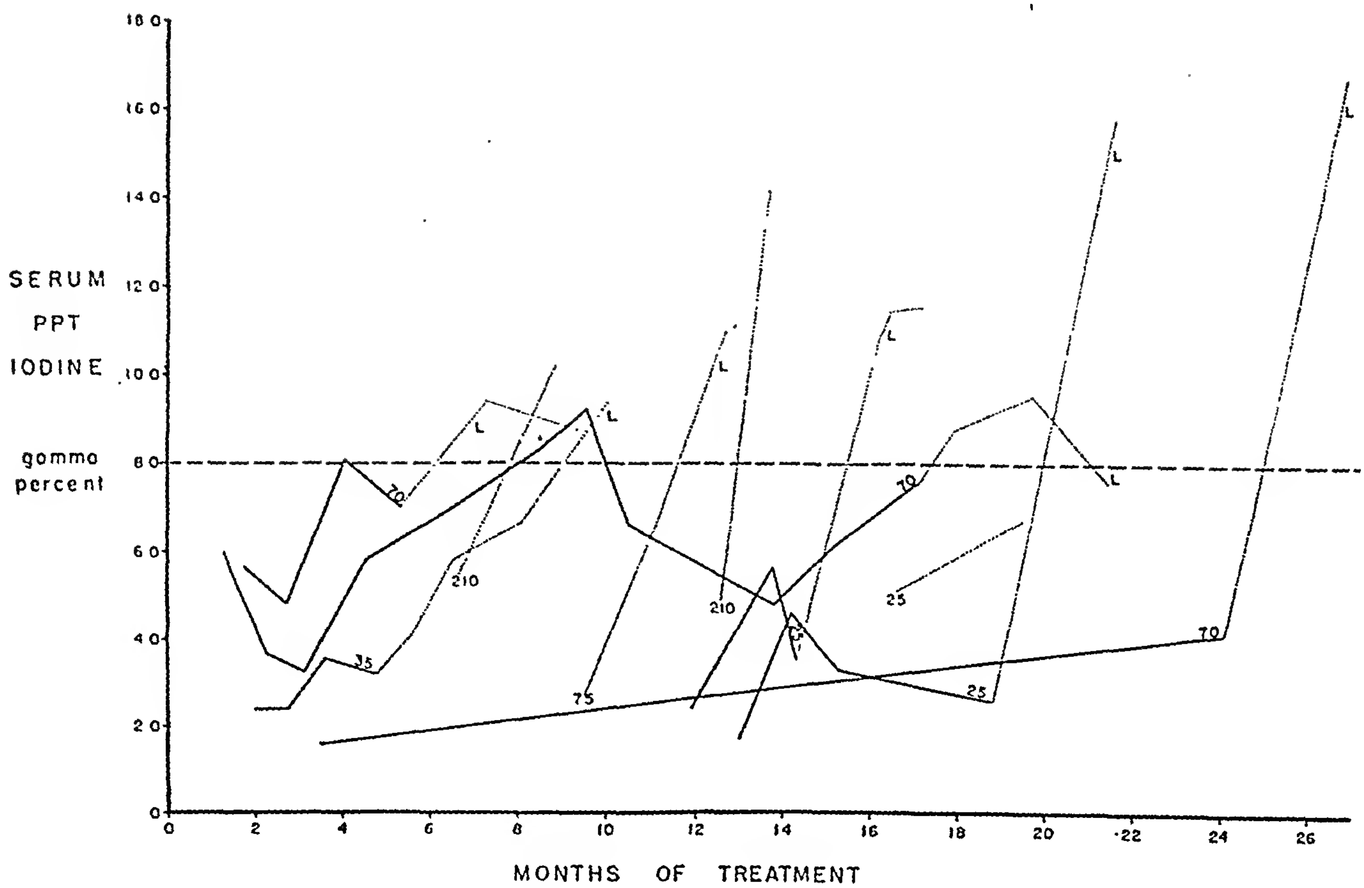

FIG. 3. EFFECT OF WITHDRAWAL OF THIOUREA

In patients in remission on thiourea plus iodine, hyperthyroidism usually recurs if thiourea medication is omitted, even after months of treatment, and even if iodine medication (all lines marked "L") is continued. The numbers refer to the daily dosage of thiourea prior to withdrawal of this medication. Dotted lines indicate changes in serum precipitable iodine following cessation of thiourea therapy.

had been repeatedly found to have euthyroid concentrations of serum precipitable iodine prior to the withdrawal of thiourea. The effect of this change in the therapeutic régime on the concentration of serum precipitable iodine is shown in Figure 3.

Hyperthyroidism recurred in 8 of the 10 patients within 6 to 12 weeks after withdrawal of the drug. This high incidence of recurrence appears to be independent of whether the iodine solution was continued or discontinued at that time.

DISCUSSION

It is evident that in most patients it is not necessary to provide a daily intake of thiourea greater than 0.07 gram given with strong iodine solution in order to produce a rapid and complete remission in hyperthyroidism. Since a response to 0.015 gram of thiourea together with iodine solution is obtained in only a very small proportion of subjects, the minimum effective dose of thiourea, given in conjunction with iodine, must lie between 15 and 75 mgm. daily in most patients. Variations in individual response make more exact estimation difficult. Two advantages may be derived from the use of smaller doses of thiourea such as 70 mgm. rather than 210 or 280 mgm. First, it is possible that the number of toxic reactions may be minimized. Although in the studies reported in this paper no untoward reactions were encountered, drug fever developed in 2 patients in a previous series (2). Second, the smaller the dose employed, the less is the probability of the development of hypothyroidism. We have, therefore, adopted the following regular plan of treatment. Ambulatory patients are usually started on 70 mgm. a day, along with strong solution of iodine, 5 drops 3 times a day. If they do not respond within 4 to 8 weeks, the dose is increased. Hospitalized patients with cardiac or other complica-

tions, on the other hand, are from the start treated with the larger amounts in addition to iodine solution. Comparison between patients receiving the entire dose once a day and those in whom it was divided and spaced in 3 parts shows no significant difference in the response of the hyperthyroidism. Divided doses are preferable, however, in those patients subject to gastrointestinal reactions.

If 210 mgm. daily are employed initially, it is desirable to reduce the maintenance dose of thiourea to 70 mgm. daily or less once remission has occurred (Table I). In this way hypothyroidism and myxedema can usually be avoided entirely. The dose should not be reduced below 25 mgm. daily since 15 mgm. and smaller amounts are generally ineffective. The large proportion of recurrence which follows the cessation of thiourea therapy (Figure 3) effectually disposes of the possibility that the hyperthyroidism has been cured.

In a previous paper (2), the additive effects of iodine and thiourea in the larger doses, 280 or 210 mgm. daily, were demonstrated. Too few observations on the effects of 70 mgm. daily doses of thiourea without iodine are as yet available to establish that this same additive effect is present at these low levels of dosage. The possibility, on the other hand, that the beneficial effects of this combined form of therapy are to be ascribed chiefly to the iodine solution given can be easily discarded. From Figure 2 it is evident that iodine solution by itself only rarely produces a sustained remission in hyperthyroidism, and from Figure 3 it is clear that iodine alone cannot sustain a remission produced by thiourea. These results confirm the general impression that continued treatment of hyperthyroidism with iodine medication alone is generally unsatisfactory.

The physiological effect of these small amounts of thiourea point to a particular sensitivity of the thyroid gland to this drug, since, at least in the normal dog, there is no tendency for the thyroid gland to destroy or concentrate thiourea in measurable amounts (7). Thiourea does not accumulate in the body to any extent and is distributed through most of the body fluids in about the same concentration as that found in serum water (7). The concentration of thiourea in serum after a daily dose of 25 mgm. is too low to measure by present analytical methods, but the general range may be estimated from the volume of distribution of thiourea (about 0.8 times the body weight) and its renal clearance (50 to 60 ml. per minute) (8). This calculation indicates that the concentration ranges from 5 to 50 gamma per cent of thiourea in body fluids including, presumably, the available water of the thyroid gland. These are to be compared with concentrations of inorganic iodine in the water of the thyroid gland of the magnitude of 10 mgm. per cent, or 10,000 gamma per cent (9). It seems improbable that relatively few molecules of thiourea can combine with and render unavailable, as has been suggested (10, 11), this great excess of inorganic iodine. The thiourea effect appears to be mediated, rather, through a block at a crucial link in the chain of reactions through which all of the iodide which is converted to organic iodine must pass. The studies reported in this paper indicate that this block in the conversion of inorganic iodine to diiodotyrosine and thyroxine can be produced by almost infinitesimal traces of thiourea.

The fact that iodine medication does not interfere with the action of thiourea, even when the latter is present in relatively trifling amounts, and the fact that their actions are additive, prove that these substances act at quite different points. There is good evidence that iodine has two separate actions on the hyperthyroid gland; the first inhibits the effect of thyrotropic hormone, the second favors the accumulation of iodine within the gland, as a result of an excess of inorganic iodine (9). Thiouracil and related drugs interfere with neither action (12 to 15). Since they certainly prevent the synthesis of thyroxine and diodotyrosine from inorganic iodine (13), it is reasonable to suggest that they may act by poisoning the enzyme system responsible for this synthesis. The lack of interference in the action of thiourea by iodine in large excess is most readily explained in this way. The experiments of Astwood (10) in which he demonstrated a greater goitrogenic potency of thiouracil in animals maintained on a low iodine diet than in those on a high iodine diet do not prove that there is a direct interference between iodine and thiouracil. The high iodine diet more probably acts by inhibiting thyrotropic hormone action and so delaying the development of goiter.

SUMMARY AND CONCLUSIONS

1. Doses of thiourea as small as 0.07 gram daily, in conjunction with iodine solution, produce a remission in hyperthyroidism with considerable regularity.

2. The remission in hyperthyroidism can usually be maintained by the use of 0.05 to 0.025 gram of thiourea daily in addition to iodine solution.

3. A daily intake of 0.015 gram of thiourea together with iodine solution does not induce a remission, or maintain it, following the use of larger amounts.

4. Withdrawal of thiourea, even when the maintenance dose is as low as 0.025 gram, is frequently followed by exacerbation of a previously controlled hyperthyroidism.

5. Control of hyperthyroidism with iodine solution alone is only rarely possible, with or without previous thiourea treatment.

6. Prolonged, and perhaps indefinite, maintenance on thiourea and iodine is necessary to prevent recrudescence of hyperthyroidism.

BIBLIOGRAPHY

1. Danowski, T. S., Man, E. B., and Winkler, A. W., Treatment of hyperthyroidism with a combination of iodine, thiourea in small doses, and desiccated thyroid. Am. J. M. Sc., 1945, 202, 777.
2. Danowski, T. S., Man, E. B., and Winkler, A. W., Additive effects of iodine and thiourea in the treatment of hyperthyroidism. J. Clin. Invest., 1946, 25, 597.
3. Danowski, T. S., Man, E. B., and Winkler, A. W., Metabolism of thiourea: additive effects of iodine and thiourea in the treatment of hyperthyroidism. J. Clin. Invest., 1946, 25, 921.
4. Danowski, T. S., and Winkler, A. W., The use of thiourea in the control of hyperthyroidism. Conn. State M. J., 1947, 11, 105.
5. Riggs, D. S., and Man, E. B., A permanganate acid ashing micromethod for iodine determinations. I. Values in blood of normal subjects. J. Biol. Chem., 1940, 134, 193.
6. Winkler, A. W., Riggs, D. S., Thompson, K. W., and Man, E. B., Serum iodine in hyperthyroidism with particular reference to the effects of subtotal thyroidectomy. J. Clin. Invest., 1946, 25, 404.
7. Danowski, T. S., Unpublished data.
8. Nicholes, H. J., and Herrin, R. C., The tubular reabsorption of urea, thiourea and derivatives of thiourea in the dog kidney. Am. J. Physiol., 1941, 135, 113.
9. Rawson, R. W., Moore, F. D., Peacock, W., Means, J. H., Cope, O., and Riddell, C. B., Effect of iodine on the thyroid gland in Graves' disease when given in conjunction with thiouracil—a two action theory of iodine. J. Clin. Invest., 1945, 24, 869.
10. Astwood, E. B., Chemotherapy of hyperthyroidism. Harvey Lectures, 1944–45.
11. Formijne, P., Probable mode of action of thiourea and thiouracil on the thyroid function. Proc. Netherlands Acad. Sci., 1946, 49, 484.
12. Larson, R. A., Keating, F. R., Jr., Peacock, W., and Rawson, R. W., A comparison of the effect of thiouracil and of injected thyrotropic hormone on the collection of radioactive iodine and the anatomic changes induced in the thyroid of the chick. Endocrinol., 1945, 36, 149.
13. Franklin, A. L., Chaikoff, I. L., and Lerner, S. R., The influence of goitrogenic substances on the conversion in vitro of inorganic iodide to thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. J. Biol. Chem., 1944, 153, 151.
14. Franklin, A. L., Lerner, S. R., and Chaikoff, I. L., The effect of thiouracil on the formation of thyroxine and diiodotyrosine by the thyroid gland of the rat with radioactive iodine as indicator. Endocrinol., 1944, 34, 265.
15. Rawson, R. W., Tannheimer, J. F., and Peacock, W. C., The uptake of radioactive iodine by the thyroids of rats made goiterous by potassium thiocyanate and by thiouracil. Endocrinol., 1944, 34, 245.

VASODILATOR FIBERS IN THE HUMAN SKIN

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Reports on vasodilator fibers in the human skin have not been in general agreement concerning either their existence or their relative functional significance (1–6). In a recent review on sympathetic surgery (7), the interruption of vasodilator fibers was cited as a possible cause for the limited benefit that occurs after sympathectomy for certain types of peripheral vascular disease. It seemed worthwhile, therefore, to reexamine the available evidence and approach the problem of vasodilator activity from points of view other than those previously described.

Lewis and Pickering (4) suggested that heating the body produces maximal vasodilatation in the skin of the extremities by inhibiting vasoconstrictor impulses and also by stimulating additional active dilatation by vasodilator impulses. If this is true, local nerve block performed during pronounced heating of the body or of the other extremities should interrupt these vasodilator impulses, exclude active vasodilatation, and produce a slight but definite fall of skin temperature. The magnitude of the reduction should be proportional to the postulated activity of the vasodilator mechanism at the time of the block.

METHOD

Patients on the surgical wards of the Massachusetts General Hospital were studied. Before beginning the experiment, the patient was placed in the cold room and covered only by a loin cloth. Skin temperatures were determined by means of 8 iron-constantan thermocouples which led to an electronic, nongalvanometric, continuous balance potentiometer.[1] This recorder registered in rotation every 30 seconds so that each thermocouple registered every 4 minutes.

When skin temperatures were determined on the hand, the palmar surface of the distal phalanx of the second and fifth digits were used. The plantar surface of the distal phalanges of the toes and a point on the lateral aspect of the dorsum of the foot were used in obtaining skin temperatures of the lower extremity. The shielded wire proximal to the naked thermocouple was taped to the appropriate site at least 2.5 cm. away from the point of actual determination so that there was no interference with heat loss at the point of determination.

Rectal temperatures, when obtained, were recorded by means of a resistance bulb and potentiometer recording on a circular chart readable to 0.02° centigrade.[2] The values at 5-minute intervals were charted. Procaine or metycaine hydrochloride was used for the local blocks. The ulnar nerve was blocked at the elbow. Ankle block was achieved by combining the conventional posterior tibial block with complete ring block at the ankle. The spinal block and the ring block of the toe were performed in the conventional fashion. Care was taken to make certain that epinephrine was not added to the anesthetic agent. In those patients in whom a sympathectomy had been done, the blocks were always performed on the intact side.

Indirect vasodilatation in the hands was induced by placing the legs in warm water at 43–44° C. up to the knees (8, 9). Vasodilatation in the feet was induced by placing the arms in warm water up to the elbows. In 1 experiment, number 4, an air envelope, through which the arms and head protruded, covered the patient, and warm air at 45° C. was blown into it. In 4 cases rectal temperature was recorded. In all cases, after placing the legs or arms in warm water, the degree of perspiration was noted.

In order to control the factor of evaporation, latex rubber was sealed over the area under observation with collodion in 2 experiments, numbers 7 and 8. In this way the loss of heat occurred from a dry surface both before and after the block.

RESULTS

Figure 1 is the chart of experiment 2 and is representative of experiments 2 to 8. The patient was brought into the cool room in which the temperature was maintained at 19.7° C. He was covered only by a loin cloth. The control temperature plateau prior to heating was obtained and at 10:30 a.m. the arms were placed up to the elbows in water at 43–44° C. Sweating began at 10:40 a.m. and soon became profuse. This was continued until a second plateau was reached and at 11:44 a.m. a complete left ankle block, including the posterior tibial nerve, was performed. Just prior to the block a towel was placed over the left foot: the artificial rise in skin temperature thus induced is indicated by broken lines in Figure 1. The

[1] Brown Instrument Co., Boston, Mass.

[2] Foxboro Instrument Co., Foxboro, Mass.

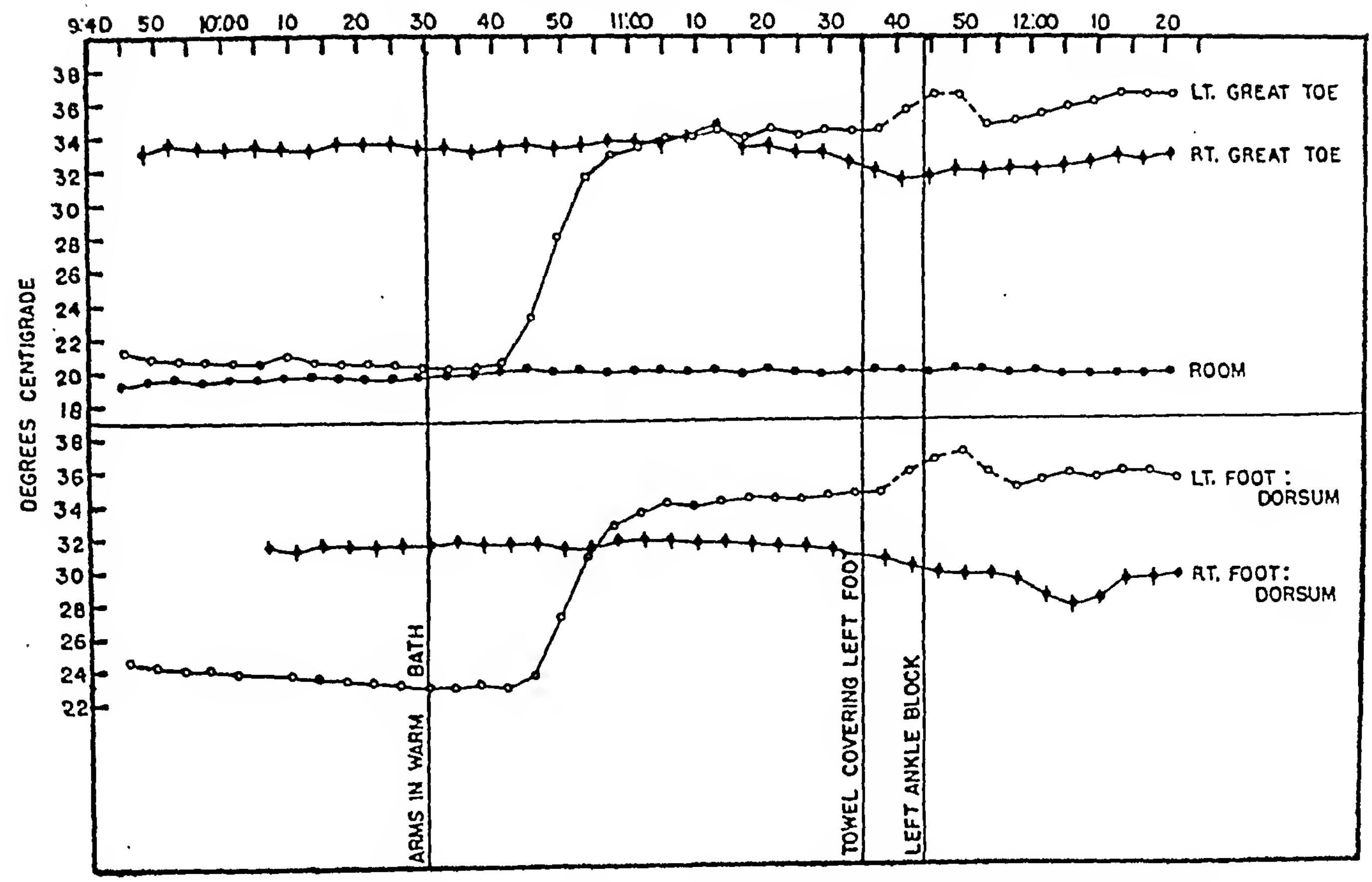

FIG. 1. PATIENT J. H., EXPERIMENT NO. 2, A RIGHT LUMBAR SYMPATHECTOMY

Arms immersed in warm bath at 10:30 a.m. Towel covering left foot produced artificial rise in skin temperature indicated by dotted lines. Ten minutes after start of ankle block, anesthesia was complete and the skin temperature of the left foot and great toe rose above the pre-block level.

towel was removed immediately and anesthesia began to set in at 11:50. This was followed by a sustained rise in skin temperature that exceeded the pre-block level by 2.1° C. on the great toe and 1.4° C. on the dorsum of the foot. A full block was still in effect at the end of the experiment.

Had active vasodilator impulses been responsible for the height of skin temperature engendered by the heating of the indifferent extremities, blocking the local nerve supply should have resulted in a fall in skin temperature. A rise actually occurred. Reflex activity in the sympathectomized extremity was apparently absent.

Table I summarizes the results. Experiment 1 was essentially a reproduction of one type of procedure upon which Lewis and Pickering based their conclusions; and, although our data are similar to theirs, we believe, as do Warren *et al*, that another interpretation is tenable. This view is supported by the results of experiments 2 to 8. Experiment 2 has already been described. In experiments 2 to 8 no evidence could be found that active vasodilators took part in the reflex vasodilatation resulting from heating the extremity or the body. Local nerve block in these experiments was followed by a rise in skin temperature instead of a fall in 5 of the 7 experiments, and in the remaining 2 the nerve block resulted in no appreciable change. In none did a fall in the skin temperature occur.

Experiments 7 and 8 represent attempts to control the factor of evaporation in order to be certain that the rise in skin temperature following the local nerve block was not due to the cessation of sweating. In these experiments the area from which the skin temperature was determined was covered with latex rubber and sealed with collodion so that heat loss occurred from a dry surface both before and after the nerve block.

Figure 2 is the chart of experiment 8. The left fifth finger was covered with a finger cot at 1 and indirect vasodilatation was induced by placing the legs in warm water at 2. Just prior to the block the patient became quite apprehensive, and

TABLE I

Response of skin temperature in normal and sympathectomized extremities to indirect vasodilatation and the response of normal extremities to indirect vasodilatation plus local nerve block

Exp. no. and patient	Disease	Room temp.	Sites of skin temperature determination	Type of block*	Skin temperature: Intact side			Sympathect. side		Rise in rectal temp.
					Before heating	During heating	After heating and block	Before beating	During heating	
		°C.			°C.	°C.	°C.	°C.	°C.	°C.
1. J. H.	Vasospasm	18.5	Great toe	None	19.2	35.8		33.2	32.3	N
			Dorsum of foot	None	21.2	35.4		32.0	31.4	
2. J. H.	Vasospasm	19.7	Great toe	P. tibial and compl. ankle	20.3	34.5	36.6	33.4	31.6	N
			Dorsum of foot		23.0	34.5	35.9	32.8	31.2	
3. E. C.	Post-polio vascular disorder	21.5	Great toe	None				34.5	34.3	2.3
			Second toe	Ring block second toe	24.5	33.7	36.0			
4. A. B.	Normal	20.0	L. 5 finger	Ulnar n. at elbow	21.3	35.7	36.1			N
			R. 5 finger	None	21.3	34.6	34.8			
5. A. B.	Normal	32.0	L. 5 finger	None		35.4	36.0			N
			R. 5 finger	R. ulnar n. at elbow		34.4	36.2			
6. P. Z.	Raynaud's	22.0	2 finger	None	23.4	32.7	33.3	29.6	27.2	0.3
			5 finger	Ulnar n. at elbow	23.1	32.8	32.9	24.5	26.2	
7. G. C.	Buerger's	20.0	R. foot dorsum	Spinal to D3	24.8	32.9†	32.9†			1.1
			L. 3 toe	Spinal to D3	22.3	24.3	25.3			
8. K. M.	Raynaud's	19.0	L. 5 finger	L. ulnar n. at elbow	20.4	32.8†	34.0†			0.7
			L. 2 finger	None	20.0	32.1	32.0			
					Before block	After block				
9. A. B.	Normal	20.4	R. 5 finger	R. ulnar n. at elbow	22.1	34.3				N
			L. 5 finger	None	22.6	22.2				

* Block always performed on intact side.
† Area covered with rubber and sealed.
N Not recorded.

a slight fall in skin temperature occurred. After the block, however, the unblocked area (the left second finger) resumed its previous level, while the blocked area exceeded its pre-block level by 1.2° C., even though the factor of evaporation was controlled throughout.

Figure 3 is the chart of experiment 7. Indirect vasodilatation was begun at 1. The dorsum of the foot was covered by latex rubber and sealed at the arrow. Spinal anesthesia was induced at 2. The arms were removed from the warm bath at 3. Despite the fact that the arterial pressure fell and

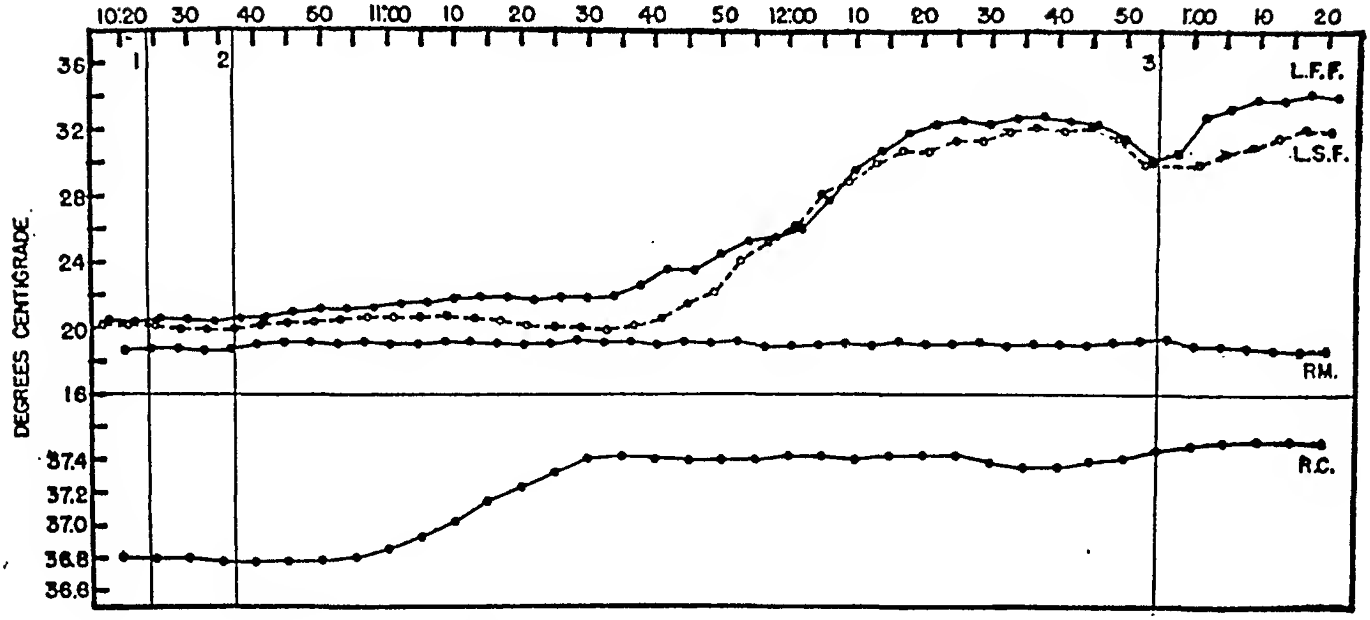

FIG. 2. PATIENT K. M., EXPERIMENT No. 8

L.F.F.—Left fifth finger (unbroken line). L.S.F.—Left second finger (broken line). 1—Left fifth finger covered with finger cot. 2—Legs immersed in warm water. 3—Procaine block of left ulnar nerve at the elbow. Patient became quite apprehensive just prior to the block, accounting for the fall in skin temperature. Following the block the anesthetized area rose to 1.2° C. above its pre-block level. The unanesthetized area resumed its previous level.

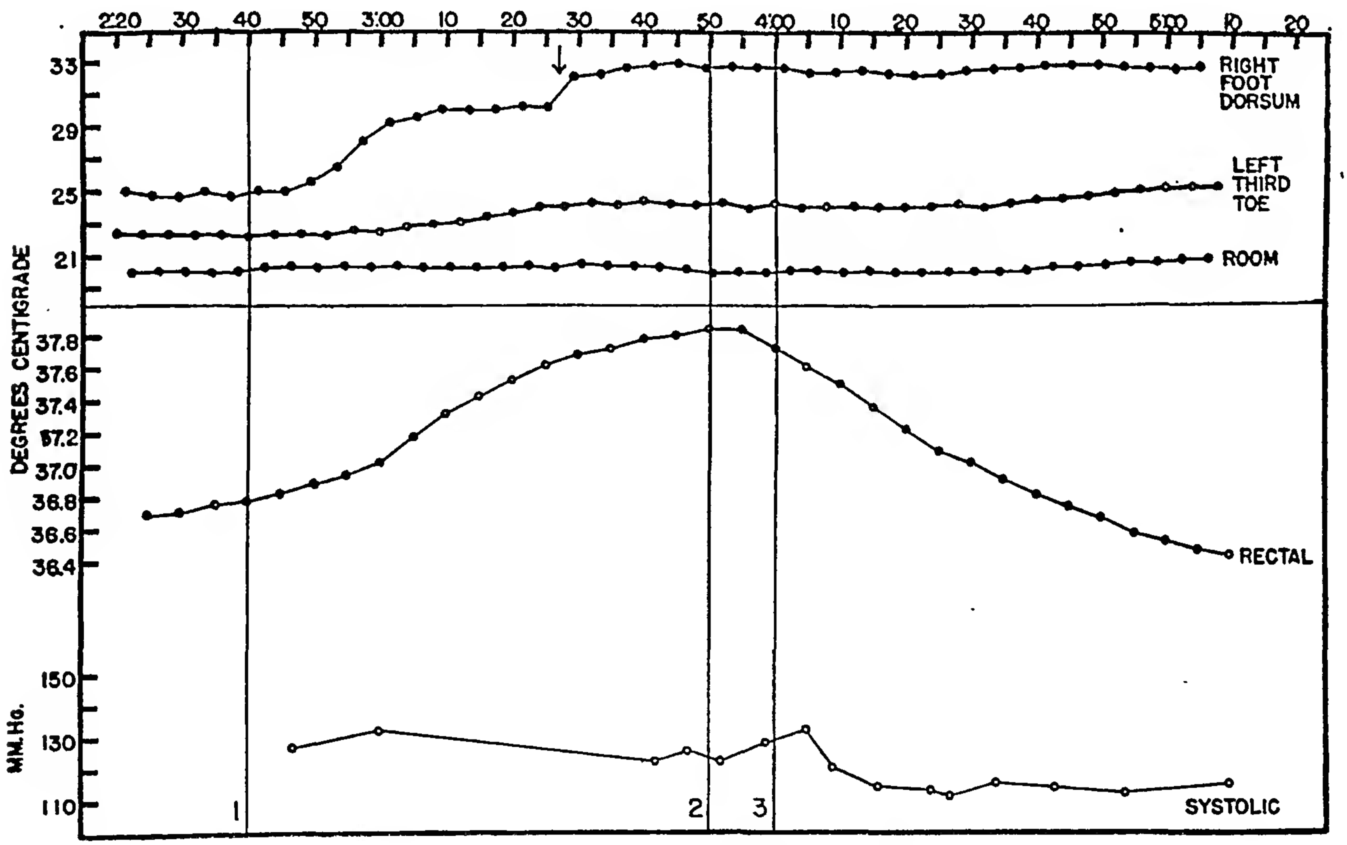

FIG. 3. PATIENT G. C., EXPERIMENT No. 7

1—Arms immersed in warm water. Arrow—right foot covered with latex rubber and collodion seal. 2—Full spinal block to D3. 3—Arms out of warm water. Despite a 1.4° C. fall in body temperature and a lower systolic arterial pressure, the skin temperature of the right foot maintained its previous level. (See text.)

there was a 1.4° C. fall in body temperature, the skin temperature of the blocked area did not fall, but maintained its pre-block level. In this experiment, as in experiment 8, the factor of evaporation was controlled by making certain that heat loss from the area occurred from a dry surface both before and after the block.

In experiments 2 to 8 inclusive (illustrated by Figures 1 to 3), "maximal vasodilatation" was first induced by heating the indifferent extremities with warm water or by heating the body with warm air. Heating was intense enough (*a*) to produce profuse sweating in every instance and (*b*) to elevate rectal temperature 0.3° to 2.3° C. in the 4 experiments in which it was determined. If vasodilator nerves share in the control of blood flow to the skin of the digits, there is every reason to believe that the stimulus used in these experiments was adequate to bring them into action. Subsequent local nerve block resulted in complete anesthesia in the appropriate area and a fall in skin temperature did not occur in any case. It is reasonable to conclude from these results that the vasodilatation produced by pronounced body warming does not include any measurable component that can be attributed to vasodilator impulses.

DISCUSSION

It is well to point out that small changes in skin temperature at the higher ranges (33° to 36° C.) reflect relatively major alterations in blood flow. Wright and Phelps (10) have found that a rise of 1° C. in this range may represent a change in the corresponding blood flow determination from 3 ml. per 100 ml. of tissue per minute to a value of 10 ml. per 100 ml. of tissue per minute.

In recent years, support for the theory that vasodilator fibers exist in the skin of the extremities comes mainly from 4 groups of investigators. Lewis and Pickering (4) performed experiments in which normal subjects and sympathectomized patients with Raynaud's disease were studied. They showed that in an individual who had undergone a dorsal sympathectomy, the temperature of the fingers of the intact hand rose to higher levels after indirect vasodilatation than did those of the sympathectomized side. They concluded from this that it was the presence of vasodilator fibers on the undisturbed side that was responsible for the higher level of skin temperature attained. Experiments were also done in which an ulnar nerve block was performed on a patient with Raynaud's disease while the patient was in a cold room. Indirect vasodilatation was then induced, and it was observed that the skin temperature of the anesthetized area did not rise while that of other areas did rise. Similar observations were made on both the lower and upper extremities (1, 2).

Grant and Holling (3) subjected patients to body heating and studied its effect on blood flow in the forearm. They concluded that the increase in flow depended upon the integrity of the sympathetic nerves and that vasodilatation was brought about both by inhibition of vasoconstrictor tone and by active vasodilator impulses. In their observations, a forearm was flushed and warmed directly and then kept so by indirect heating. Local nerve block then caused the anesthetized area to pale, cool, and cease sweating. However, as pointed out by Warren, *et al* (6), the authors are unable to account for the difference between the effect of sympathectomy which increases forearm flow and the effect of local nerve block which, they concluded, under certain conditions, diminishes it. It should also be added that these investigators added epinephrine to the procaine with which the local nerve blocks were performed. Similar experiments were performed by Doupe *et al* (11).

Warren *et al* (6) also point out that when body heating produces indirect vasodilatation which is greater in the intact than in the sympathectomized extremity, this cannot be used as evidence for the presence of vasodilator fibers, for it has been repeatedly demonstrated that the peripheral vascular bed of a sympathectomized extremity acquires an appreciable tone and that this tone is not affected by efforts at homeostasis mediated through the central nervous system. We have in fact observed several individuals in whom a unilateral sympathectomy had been performed and in whom a "paradoxical response" was observed, that is, when indirect vasodilatation was induced, the temperature of the sympathectomized extremity fell several degrees while that of the intact side responded in the usual fashion. This phenomenon, produced experimentally, is accompanied by a slight lowering of the arterial pressure.

Warren *et al* (6) have demonstrated that complete procainization of the sympathetic supply to the upper extremity causes an increase in blood

flow that is equivalent to the maximal increase produced by immersing the hand in water at 43° C. plus indirect heating. The authors concluded that these data make it unnecessary to assume the presence of active vasodilator fibers to the skin.

In the various investigations which purport to demonstrate the functional activity of vasodilator fibers, the lack of a rise in skin temperature following local or paravertebral block plus indirect heating was construed as being due to a lack of vasodilator activity in the anesthetized area. These experiments were made on patients suffering from vasospastic disease. Yet we know that where diminished peripheral flow is due to arteriolar constriction alone, block of the nerve supply to that area is followed by a local rise in skin temperature.

In this connection it is profitable to examine the chart of patient number 7 (Figure 3). At point 1, the arms were placed in warm water and the skin temperature on the dorsum of the right foot rose while that of the left third toe did not. Had a procaine block of the left third toe been performed prior to 1, this curve would resemble that type of chart upon which the main body of evidence in favor of vasodilator fibers in the human skin is founded. That the block was not performed indicates that other factors may contribute to this type of result. Support is given this view by the fact that this result has not been obtained in the normal human, but only in the patient with well established peripheral vascular disease. It is of additional interest to note that 8 days after a left lumbar sympathectomy, the skin temperature of the left third toe was 30.4° C. after the patient had been exposed for 1 hour in the cold room.

It cannot be denied that, prior to the block, when the skin temperature is high due to indirect heating, there may remain a certain amount of vasoconstrictor plus vasodilator tone, if such exists. In this case, the release of the residual vasoconstrictor tone may overbalance the release of vasodilator tone resulting in a net rise in skin temperature. It is for this reason that we have not found it possible to devise a conclusive experiment which would demonstrate that vasodilator fibers to the human skin do not exist. It was believed, however, that the above experiments do strongly suggest 2 facts: first, that previous investigations have not established the functional activity of vasodilator fibers in the hand or foot; second, that, if they do exist, their functional significance is very limited.

It is not the purpose of this report to consider the controversy as to whether or not active vasodilator impulses travel in posterior root fibers. After careful study, Westbrook and Tower (2) concluded, "The concept that nerve fibers emerge from the spinal cord into the posterior roots in adult mammals including man is without foundation in anatomical fact or physiological necessity and therefore may be dispensed with." Whether this point of view becomes generally recognized as correct is not important to the issue involved in this study. The nerve blocks performed in these experiments were either subarachnoid or peripheral somatic and therefore should block all impulses going to the area under examination whether they travel via the somatic or autonomic pathways.

Additional evidence indicating that active vasodilator fibers traveling in somatic nerves are of little or no importance in elevating the skin temperature consequent to indirect vasodilatation may be seen in Figure 1. The right lumbar sympathectomy had interrupted the sympathetic pathways supplying this patient's right lower limb. The absence of any reflex change in that extremity is evidence that the remaining nerves supplying that extremity were inactive in this regard. The same considerations apply to experiments 1, 3, and 6. Curves of this type are commonly seen in the literature on peripheral vascular disease (2, 4, 5). Further evidence supporting this view may be found in the work of Dole and Morison (13).

These considerations have clinical as well as physiological implications. Grimson, in a recent, extensive review on the limitations of sympathectomy as a therapeutic procedure (7), cites the possibility that the removal of vasodilator fibers may account for the lack of benefit in some instances. We believe that in the normal human, as well as in the patient with well established Raynaud's or Buerger's disease, vasodilator fibers are of only slight significance at most. This factor, therefore, should not be allowed to enter into the consideration of whether sympathectomy should

be performed in any given case, insofar as the circulation to the skin of the hands and feet is concerned.

SUMMARY

Experiments have been performed to test the theory which holds that vasodilatation in human skin in response to the heating of the indifferent extremities is due in part to vasodilator impulses. It was found that at the height of the elevation of skin temperature due to reflex vasodilatation, block of the nerve supply to that area did not cause a fall in skin temperature. In most cases an elevation occurred. These observations were made upon patients with a normal peripheral vascular bed as well as in patients with Raynaud's and Buerger's disease.

It may be concluded that if active vasodilator fibers exist in the skin of the digits their functional significance is very limited. The indirect vasodilatation produced by heating the indifferent extremities is, therefore, due to the central inhibition of vasoconstrictor impulses. Evidence was cited to indicate that these considerations apply to both somatic and autonomic pathways.

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BIBLIOGRAPHY

1. Fatherree, T. J., The mechanism of indirect vasodilatation induced by heat, and evidence of sympathetic vasodilator nerves in man. Proc. Staff Meet., Mayo Clin., 1938, 13, 508.
2. Fatherree, T. J., and Allen, E. V., Sympathetic vasodilator fibers in the upper and lower extremities; observations concerning the mechanism of indirect vasodilatation induced by heat. Arch. Int. Med., 1938, 62, 1015.
3. Grant, R. T., and Holling, H. E., Further observations on the vascular responses of the human limb to body warming; evidence for sympathetic vasodilator nerves in the normal subject. Clin. Sc., 1938, 3, 273.
4. Lewis, T., and Pickering, G. W., Vasodilatation in the limbs in response to warming the body; with evidence for sympathetic vasodilator nerves in man. Heart, 1931, 16, 33.
5. Richards, R. L., The Peripheral Circulation in Health and Disease. E. & S. Livingstone Ltd., Edinburgh, 1946.
6. Warren, J. V., Walter, C. W., Romano, J., and Stead, E. A., Jr., Blood flow in the hand and forearm after paravertebral block of the sympathetic ganglia. Evidence against sympathetic vasodilator nerves in the extremities of man. J. Clin. Invest., 1942, 21, 665.
7. Grimson, K. S., Sympathectomy and the circulation—anatomic and physiologic considerations and early and late limitations. Surgery, 1946, 19, 277.
8. Gibbon, J. H., and Landis, E. M., Vasodilatation in the lower extremities in response to immersing the forearms in warm water. J. Clin. Invest., 1932, 11, 1019.
9. Landis, E. M., and Gibbon, J. H., Jr., A simple method of producing vasodilatation in the lower extremities with reference to its usefulness in studies of peripheral vascular disease. Arch. Int. Med., 1933, 52, 785.
10. Wright, G. W., and Phelps, K., A comparison of procedures for increasing blood flow to limbs using an improved optical plethysmograph. J. Clin. Invest., 1940, 19, 273.
11. Doupe, J., Cullen, C. H., and Macaulay, L. J., Studies in denervation; circulation in skin of proximal parts of limbs. J. Neurol. & Psychiat., 1943, 6, 129.
12. Westbrook, W. H. L., Jr., and Tower, S. S., Analysis of problem of emergent fibers in posterior spinal roots, dealing with rate of growth of extraneous fibers into roots after ganglionectomy. J. Comp. Neurol., 1943, 72, 383.
13. Dole, V. P., Jr., and Morison, R. S., A note on the question of reflex activation of dorsal root dilators. Am. J. Physiol., 1940, 130, 304.

THE EFFECT OF VARYING PULMONARY PRESSURE ON THE ARTERIAL PRESSURES IN MAN AND ANESTHETIZED CATS [1]

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Numerous observations of the effects of pressure breathing on the arterial blood pressure of anesthetized animals are available in the literature (1–7). In all cases an increase in the pulmonary pressure results in a fall of the arterial pressure (mean pressure as measured on a mercury manometer), with an occasional secondary compensatory rise.

With negative pulmonary pressures the effects are less regular, according to Schaefer and Bain (2), but usually the arterial pressure rises (7). Bruns (8) showed an increased stroke volume in a plethysmographic record of a rabbit heart during negative pressure breathing and Böger, Cobet, and Stepp (9) found an increased cardiac output of 15 to 162 per cent, and an increased oxygen consumption (37 per cent) with a negative pulmonary pressure of 5 cm. H_2O in a rabbit. Since these experiments were done on anesthetized animals it is difficult to apply them directly to the human problem.

Most measurements previously made on men are confined to brief periods of increased positive pressure such as may be obtained by the Valsalva experiments (5, 6, 10–14). It has been generally found that with such an increase of intrapulmonary pressure the systemic arterial pressure rises briefly and then falls. This is followed by a secondary rise above the initial level. The secondary rise does not occur if the intrapulmonary pressure is too high. The heart rate usually accelerates. According to Bürger (13), only 3 out of 145 cases showed a slowing of the heart. Barach *et al* (15) recently reported a series of experiments on men breathing air at a constant pressure of 20 cm. H_2O in which he found a rise in arterial blood pressure equal approximately to the applied pulmonary pressure.

The effects of negative pressures in man were studied by Kuhn (16, 17) who used a mask (Saugmaske) with a restricted inlet orifice and a large outlet. Because of the increased amount of blood in the lungs this was regarded as a useful type of therapy for tuberculosis. Evidence was also offered however to show that the volume of the pulse was increased. Later, Bruns (8, 18) concluded, mostly on the basis of similar evidence, that negative pulmonary pressure increases the cardiac output. He recommended negative pressure breathing for patients with poor venous return and certain types of heart disease. Subsequent investigators have paid little attention to this subject and the treatment has not been further investigated possibly because of the danger of dilating the heart or rupturing a vein in the thorax. One such case of dilated heart was recorded by Stigler (19) as a result of negative pressure breathing (man under water breathing surface air through a tube). The evidence concerning the physiological effects of this procedure in man is still fragmentary.

The experiments reported below were designed primarily to elucidate the problems of positive pressure breathing of possible military importance, but for the sake of completeness they were extended to cover negative pressures as well. The respiratory effects of these same procedures on our subjects were studied simultaneously and the results have been reported elsewhere (20).

METHODS

For the animal experiments, cats were anesthetized with dial. Mean arterial pressures were obtained by a carotid

[1] This work was done under contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Rochester. The data included in this paper were obtained by the cooperative effort of a large group including Doctors Hermann Rahn, L. H. Chadwick, Arthur B. Otis, Lorin J. Mullins, R. E. Gosselin, and others who kindly served as subjects. Some of the other data from the same experiment have been published elsewhere (Rahn, Otis, *et al*, 1946 and Otis, Rahn, *et al*, 1946).

artery cannula connected to a recording mercury manometer. Simultaneous right auricular pressures were recorded by means of a sound inserted to the level of the heart through the jugular vein, activating a recording tambour. Peripheral venous pressures were measured on a saline manometer connected to a femoral vein cannula. The tracheal cannula was attached to a very short sidearm on a large rubber hose. One end of the hose was connected to a compressed air line for positive pressures or to a vacuum pump for negative pressure. In either technique air was passed through the hose rapidly enough to avoid rebreathing. Pressures were regulated by a pinch clamp on the end of the hose opposite to the pump. Intratracheal pressures were measured on a water manometer connected to a sidearm in the cannula. This system permitted the pulmonary pressure to be set at any value up to 40 cm. H_2O, positive or negative, and the variation in pressure during the respiratory cycle was not over ± 3 cm. H_2O. The animal board was constructed so that the animal could be tilted either head or feet down (45 degrees) without altering the position of the heart relative to the recording instruments.

In the experiments on humans, systolic and diastolic pressures were read partly by the usual auscultatory method but more often by the use of the Tycos recording sphygmomanometer. Heart rates were counted from the carotid pulse over a 30-second interval. Measurements were made, with the subjects in the supine and in the sitting positions. In the lying position, the subjects were in a Drinker respirator and the equivalent of a positive pulmonary pressure was obtained by a continuous negative pressure in the respirator. For the sitting position we used a "body box" with the head protruding through a rubber collar in the top, or a helmet with the bottom closed by a similar collar. When the subject was in the body box or the Drinker respirator the blood pressure in the arm was recorded outside the box. In this case the pressure as read was the pressure in relation to the pulmonary or atmospheric pressure as zero, not the pressure as related to that surrounding the arm. For the sake of uniformity, therefore, we added the differential pulmonary pressure to our readings. In the charts all the pressures are those which would be read in the arm in the usual way if the positive pressure had been applied to the head only by means of a helmet. The usual procedure was to increase or decrease the pulmonary pressure in steps of 8 mm. Hg, holding the pressure at each stage for from 5 to 8 minutes.

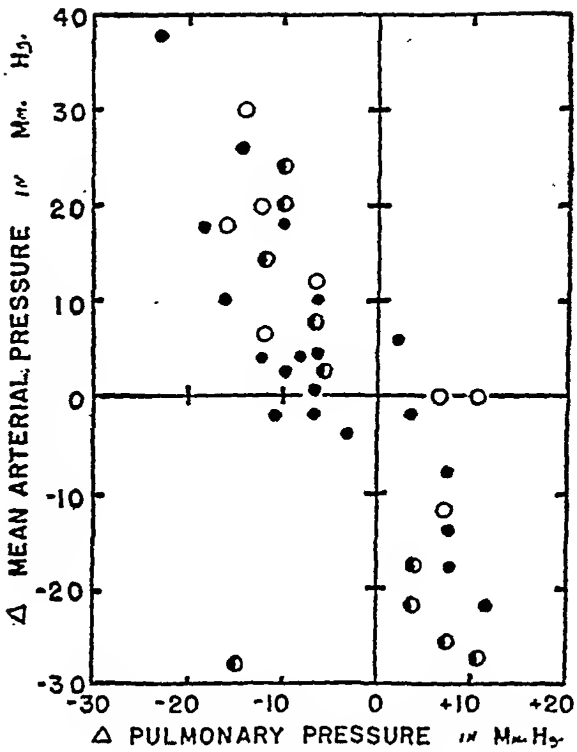


FIG. 1. THE CHANGE IN THE MEAN ARTERIAL PRESSURE OF THE CAT WHEN THE INTRAPULMONARY PRESSURE IS ALTERED

Ordinates: The change in mean arterial pressure in millimeters of mercury.

Abscissae: The change in intrapulmonary pressure in millimeters of mercury.

Meaning of symbols: Open circles: Cat in head-down position. Half closed circles: Cat in feet-down position. Solid circles: Cat in horizontal position.

RESULTS

(1) *Cat experiments.* The data of 4 cat experiments are given in Figure 1. Abscissae represent the applied intrapulmonary pressure in mm. Hg; and the ordinates, the change in mean arterial pressure. Each determination with negative pressures was made over an interval of from 1 to 5 minutes. The determinations with positive pressures are open to question since each application was brief due to threatened respiratory embarrassment and adequate time was probably not allowed for the compensatory rise found by others (2). With one exception negative pressures gave a rise in arterial pressure. With positive pressures, there is a tendency for the feet-up position to protect against a fall in arterial pressure and for the feet-down position to render the animal more susceptible to this effect. If true, this is presumably due to the effect of these positions on the venous return. The effect of negative pressures appears to be independent of the position of the animal. During the course of an experiment there was a slow but steady decline in arterial pressure. It was observed that negative pressures produced a greater rise in blood pressure if the arterial pressure was initially low, in general tending to restore the arterial pressure to normal. Shortly before

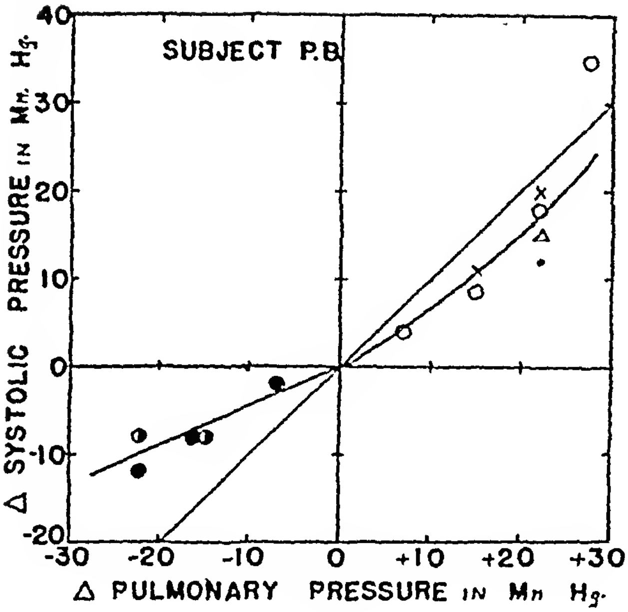


FIG. 2

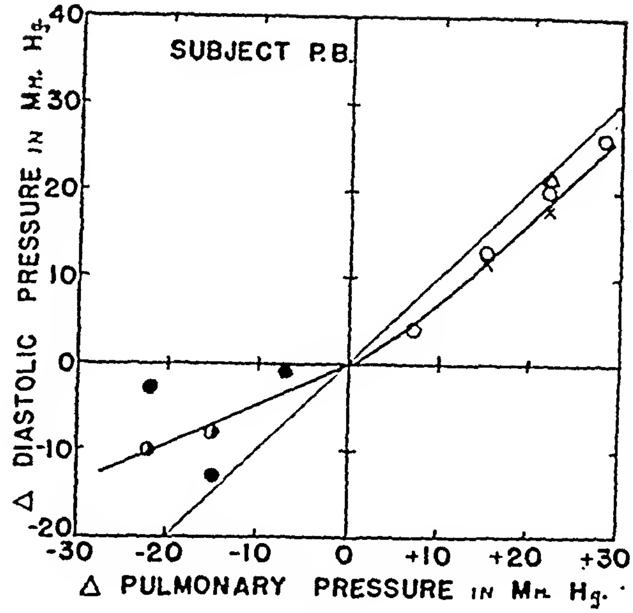
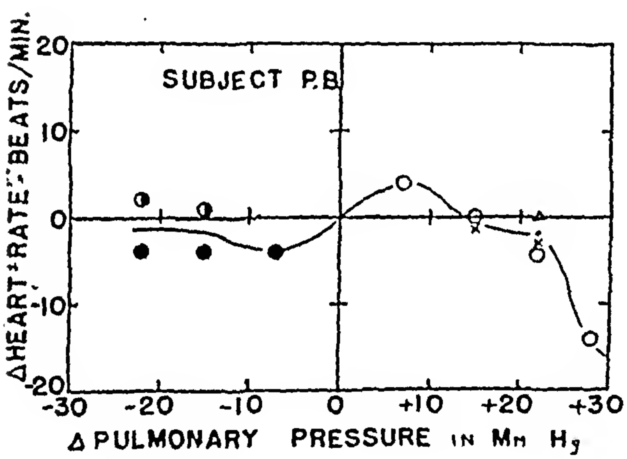


FIG. 3



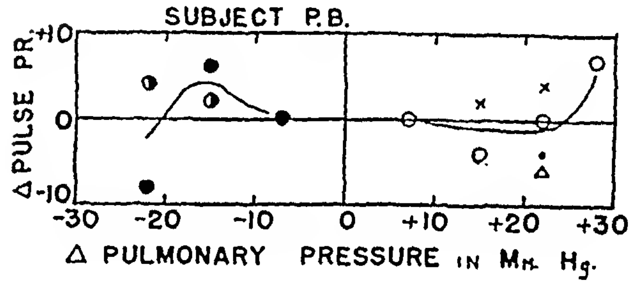


FIG. 4

FIG. 5

FIGS. 2 through 5. The changes in systolic blood pressure in mm. Hg, diastolic blood pressure in mm. Hg, the pulse pressure in mm. Hg, and heart rate in beats per minute for one subject in the sitting position in response to changes in intrapulmonary pressures. Abscissae: The change in intrapulmonary pressure in mm. Hg.

death, however, negative pressure failed to alter the arterial pressure.

Our venous pressure data in the cat will not be presented in detail because they confirm, in general, those of Holt (21). In both cases the auricular pressure falls by an amount equal to the decrease in pulmonary pressure.[2] With increased pulmonary pressures in the dog, Holt found a rise in auricular pressure almost equal to the rise in applied pressure, but in our experiments on cats the rise was only about ½ that of the pulmonary pressure. Holt (22) and Otis *et al* (23) in their studies on man report a 50 per cent rise. The failure to obtain a greater rise has been explained by the absorption of part of the applied pulmonary pressure by the elasticity of lung tissue.

(2) *Experiments on man.* Typical results on one individual, P. B., in the sitting position are shown in Figures 2 through 5. The changes in systolic pressure are plotted against the changes in the pulmonary pressure. It is evident that the systolic pressure rose with positive and fell with negative pulmonary pressures. A change in the blood pressure equal to the change in the pulmonary pressure is indicated by the straight diagonal

[2] This equality of pressures appears to indicate that the lung is more distensible when the volume and pressure are low than in the normal breathing range.

lines passing through the origin. Since all (but one) of the points are below this line during positive pressure breathing it is evident that the systolic pressure does not follow completely the change in pulmonary pressure. However, the intrathoracic pressure does not rise quite so much as the pulmonary pressure because of the elasticity of the lungs; so it is probable that in this case the systolic pressure is closely following the intrathoracic pressure. The changes in the heart rate of P. B. (Figure 4) were variable. On some days it increased and on other days it decreased under apparently comparable conditions. Similar variations were occasionally observed in other subjects.

In Figures 6 and 7 are shown curves for systolic pressures in other individuals in the sitting and the

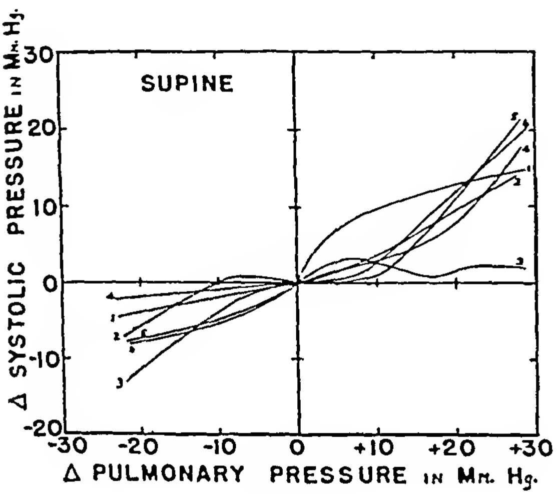


FIG. 6

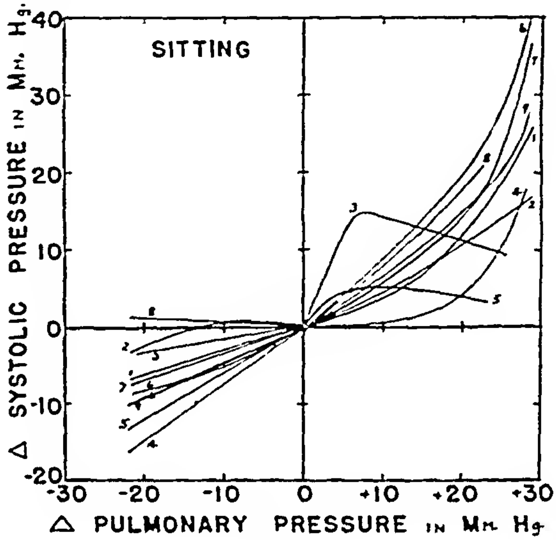


FIG. 7

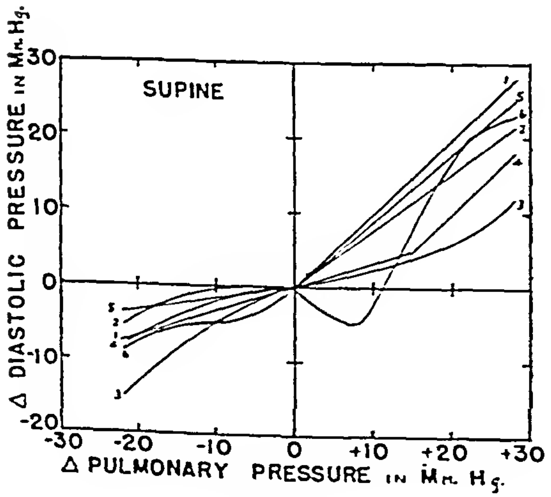


FIG. 8

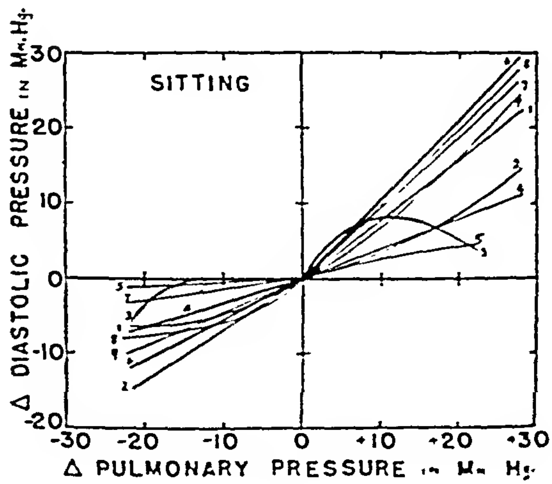


FIG. 9

Figs. 6 through 9. The changes in systolic and diastolic blood pressure in response to a change in applied pulmonary pressure. Figures 6 and 8 are for the supine position; Figures 7 and 9 are for the sitting position. Ordinates: Figures 6 and 7, the change in systolic pressure in mm. Hg. Figures 8 and 9, the change in diastolic pressure in mm. Hg. Abscissae: The change in applied pulmonary pressure in mm. Hg. Each curve is for a single subject. Numbers on the graph identify the different subjects.

supine positions respectively. The numbers on the curves refer to the numbers of the subjects. Different subjects were used however for the two series so that similar numbers in the two series do not refer to the same subjects. All the subjects were members of the staff or medical students, and all were in normal health. The corresponding diastolic pressures in these same experiments are shown in Figures 8 and 9. The numbers of the subjects in Figures 6 and 8 correspond, as do those in Figures 7 and 9. The general result obtained in all these experiments is very evident. Positive pressures increase and negative pressures decrease both systolic and diastolic pressures. An occasional exception occurs.

The curves in Figures 6 to 9 were drawn to give a reasonable fit to the scatter of points obtained with each individual as indicated for P. B. in Figures 2 to 5. To avoid confusion the individual points are not given. Details of the curves often represent only single points and are of no significance. At the highest pressures of 28 mm. Hg the systolic pressures seem unduly high in many cases. We did not usually leave the subjects very long at this pressure and the high reading may be only an initial effect due to the excitement. When the pulmonary pressure reaches a point where the venous filling pressure is no longer able to compensate, the arterial pressure would of course be low. In subject No. 3 in the sitting position (Fig-

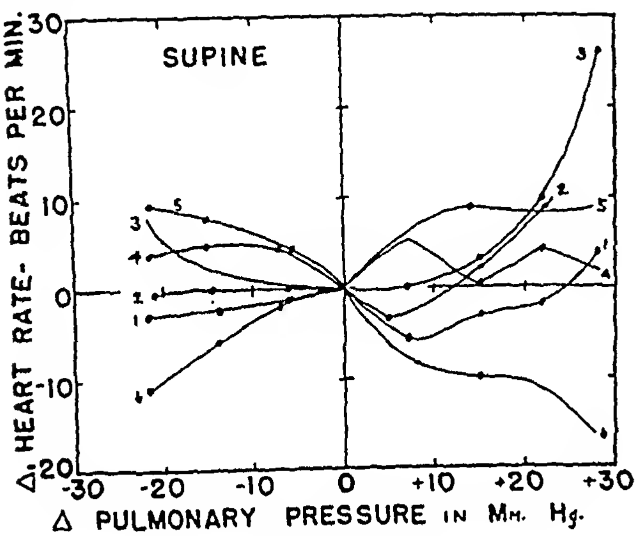


FIG. 10

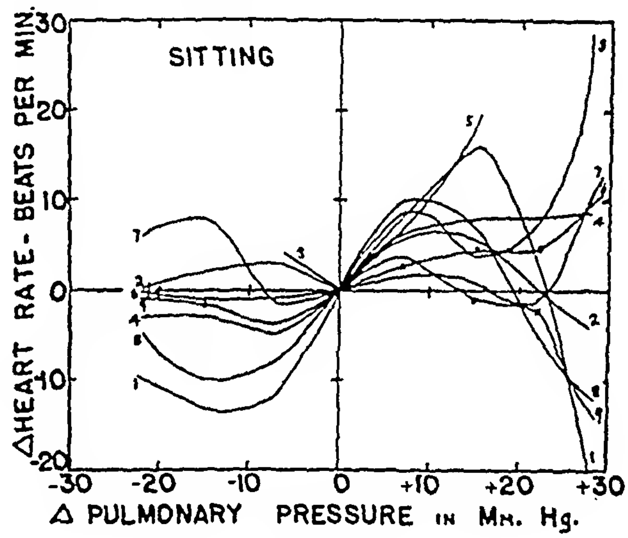


FIG. 11

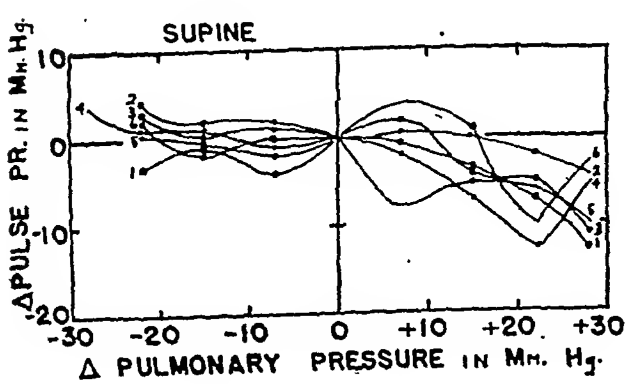


FIG. 12

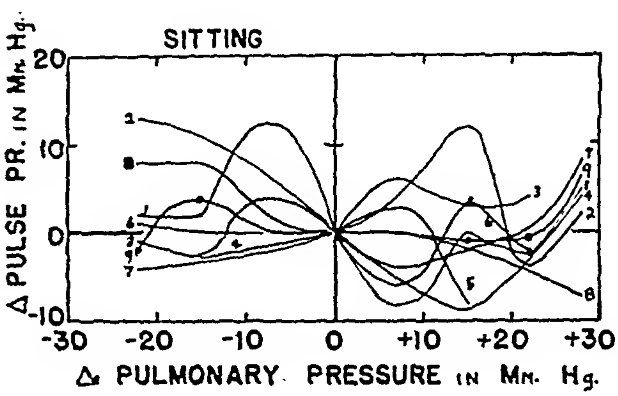


FIG. 13

FIGS. 10 through 13. The change in heart rate and pulse pressure in response to a change in applied pulmonary pressure. Figures 10 and 12 are for the supine position; Figures 11 and 13 are for the sitting position. Ordinates: Figures 10 and 11, the change in heart rate in beats per minute. Figures 12 and 13, the change in pulse pressure in mm. Hg. Abscissae: The change in applied pulmonary pressure in mm. Hg. Each curve is for a single subject identified by the number on the graph.

ures 7 and 9) we actually found an arterial pressure too low to give any readable deflection in the record. This occurred just before the subject had to be relieved of the pressure because of a threatened collapse. It was generally observed that the subject's blood pressure reached an equilibrium for a given pulmonary pressure within 1 minute after its application. It was also noted that in those subjects who nearly collapsed the blood pressure was maintained at its normal level until a minute or two before the onset of severe distress

A comparison of Figures 6 and 7 shows that with positive pressures the systolic blood pressure rises to a higher level in the sitting than in the supine position for an equal increment of pulmonary pressure. However, the average diastolic pressures for both positions follow roughly the intrathoracic pressure although the variation is large. This results in a decrease in the average pulse pressure in the supine position but no change in the sitting position. The various explanations suggested for these facts may be omitted here as purely speculative.

Changes in heart rate in the sitting and the supine positions are shown in Figures 10 and 11 respectively. The numbers represent subjects as before. Numbers in Figures 10 and 12 correspond to those in Figures 6 and 8. In these figures the graphs are drawn through the actual points or the average points (where 2 or more observations were available). Such average points are indicated by dots on the curves. Detailed variations are of no significance, but it may be noted that, in the sitting position especially, there is a rather pronounced tendency in some subjects toward a decreased heart rate at the higher pressures. When the subject became alarmed at these high pressures or was about to collapse the rate might have gone high. (See curves for subject No. 3 in Figure 11.) Low heart rates have usually been attributed to increased intracerebral pressure and the absence of an acceleration due to excitement and effort or to a less sensitive carotid sinus which would not respond to a decreased pulse pressure. The incidence of low heart rates with increased pressure is much higher in our series than that reported by Bürger (13). Most individuals gave the same characteristic response on successive days, either a rise or a fall, but some gave an increase one day and decrease the next without apparent cause.

In the sitting position, at least, there is no consistent change in pulse pressure as can be seen from the average curves for 8 subjects shown in Figure 13. In the supine position however there appears to be a slight decrease of 10 or 15 per cent at the extreme pressure of 30 mm. Hg (Figure 12). This is consistent with the observation of a slightly decreased cardiac output (24).

DISCUSSION

Since our results with human subjects show that the arterial pressure follows approximately the intrapleural pressure (whether positive or negative) it may be argued either (1) that the explanation is purely mechanical, *i.e.* the arterial pressure is higher simply because the initial pressure in the ventricle was higher before systole began, or (2) that the blood pressure level is regulated reflexly by afferents arising from structures such as those around the aorta, which are exposed to the variations in intrapleural pressure. Such structures would respond in such a way as to maintain a constant difference between arterial and intrapleural pressure. Central structures within the brain itself would presumably behave in the same way since the pressure of the cerebrospinal fluid varies closely with the intrapleural pressure (Hamilton, Woodbury and Harper, 25). In our experiments with either helmet or body box the carotid sinus was exposed exteriorly to a pressure equal to the pulmonary pressure. If this organ were solely responsible for the regulation of the level of the arterial pressure, it would have kept the level of the blood pressure constant relative to the pulmonary pressure. Actually, the blood pressure is more nearly constant relative to intrathoracic pressure which increases somewhat more than half as much as the pulmonary pressure. Furthermore, Barach *et al* (15) have shown that when pressure is applied by mask, the arterial pressure remains constant relative to the pulmonary pressure. With their technique, the carotid sinus is not affected by the applied pulmonary pressure but is exposed through the skin to atmospheric pressure at all times. Its presumed effect would therefore be to control the arterial pressure at a level which is constant relative to atmospheric pressure. Since, in both techniques, the expected carotid sinus effect is apparently absent, it may be concluded that the carotid sinus is in-

sensitive to a rise in arterial pressure of at least 15 mm. Hg or to a fall of at least 10 mm. Hg, or that it is dominated by other receptors.

The ability of an individual to maintain pulmonary pressure has been used as a test of right ventricular fitness (6, 10, 11, 13, 26) on the theory that pressure increases the resistance of the pulmonary circuit. This is borne out by the work of Heinbecker (27) who perfused lungs in an artificial thorax. When they were inflated by negative pressure in the thorax, the flow increased but when they were inflated by positive intrapulmonary pressure the flow was decreased. No heart was included in this circuit so that inflow and outflow pressures were constant, and the result can clearly be attributed to the enlargement or diminution in the size of the vessels due to the respective increase or decrease in mean pressure surrounding them. When the heart is included in the circuit the situation is more complex because the heart is also exposed to the intrathoracic pressure. The immediate effect of pressure changes on the flow depends upon the capacity of the lungs (28, 29) which varies greatly with pressure, but in general the decrease in pulmonary resistance with negative pressure inflation is confirmed (30). In these experiments, however, the venous pressure remained constant while in the body this is not the case and the flow through the pulmonary circuit depends more upon the filling of the heart than upon the pulmonary resistance. This is especially true when the intrapulmonary pressure is maintained higher or lower than normal more or less indefinitely so that the capacity of the lung vessels can reach a steady state value.

The filling of the heart depends of course upon the venous pressure which in man appears to follow the intrapleural pressure with remarkable promptness and fidelity (23). With positive pressure the venous return is therefore well maintained although there is evidence of a slight decrease in cardiac output (15, 24). This is indicated indirectly also by the rise of arterial pressure in man with increased intrapulmonary pressure. In the anesthetized cats, however, where the arterial pressure fell, the inflow into the heart must have diminished as it does in anesthetized dogs (31). Conversely in the cats a rise of arterial pressure was observed with negative pressure breathing indicating that the venous return was improved. In man the absence of such an increase in arterial pressure suggests that the normal venous pressure was adequate to fill the heart to its limit so that further increases of the pressure gradient had no further effect. The fall in arterial pressure which we have observed in man with negative pulmonary pressures lends no support to the idea that this procedure increases cardiac output and would therefore be beneficial, but it does not contradict this possibility. Since we have observed a diminished pulse volume during positive pressure breathing it seems probable that an increase in pulse volume occurs in negative pressure breathing as has previously been reported (17, 8, 32). Böger *et al* (9) found the heart rate decreased in anesthetized rabbits but Bruns (18) found it mostly increased in man. It is possible, therefore, that with negative pressure breathing there is a slight increase in the cardiac output just as, with positive pressure, there is, if anything, a slight decrease (15, 24). Such an increase would be particularly likely to occur in any condition in which the venous return was initially defective. Further work on the effects of negative pulmonary pressures is needed to verify this interpretation of our results.

SUMMARY

1. The response of the systolic and diastolic pressures and heart rate of man, and of the mean arterial, right auricular, and femoral venous pressures of the anesthetized cat, to increased and decreased intrapulmonary pressure was studied. Body position was also varied.

2. In the cat, mean arterial pressures rise in response to a decreased pulmonary pressure, independently of the position of the animal. With increased pulmonary pressure only the primary fall of arterial pressure was studied, and this was much less in the head down than in the feet down position. Holt's (21) venous pressure observations were confirmed.

3. In man, in both supine and sitting positions, increased intrapulmonary pressure gave an increase in both systolic and diastolic pressures, and decreased intrapulmonary pressure gave a fall in both.

4. Pulse pressures in many were variable ($\pm$ 10 mm. Hg) and tended to give a consistent decrease only with positive intrapulmonary pressure in the supine position.

5. Heart rates were variable (±10 beats per minute).

6. If increased intrapulmonary pressure produced circulatory failure, arterial pressures and heart rates were maintained until within a minute or two of the onset of severe symptoms.

7. The effect of pressure breathing on blood pressure regulatory receptors is discussed.

BIBLIOGRAPHY

1. Chillingworth, F. P., and Hopkins, R., Physiologic changes produced by variations in lung distention. Am. J. Physiol., 1920, 51, 289.
2. Schafer, E., and Bain, W. A., The effects of changes in intrapulmonary air-pressure on the pulmonary and aortic circulation of the dog. Quart. J. Exper. Physiol., 1932, 22, 101.
3. Humphreys, G. H., Moore, R. L., and Barkley, H., Studies of the jugular carotid and pulmonary pressures of anesthetized dogs during positive inflation of the lungs. J. Thoracic Surg., 1939, 8, 553.
4. Ferguson, J. K. W., Changes in respiration on inflation and deflation of the chest. Am. J. Physiol., 1940, 130, 675.
5. Bruck, E., Über den Blutdruck bei plötzlichen starken Anstrengungen und beim Valsalvaschen Versuch nebst Untersuchungen über die hierbei eintretenden Veränderungen der Herzgrösse. Deut. Arch. f. Klin. Med., 1907, 91, 171.
6. Bürger, M., Über die Bedeutung des intrapulmonalen Drucks für den Kreislauf und den Mechanismus des Kollapses bei acuten Anstrengungen. Klin. Wochenschr., 1926, 5, 777 and 825.
7. Rost, E., Beitrag zur Kenntnis der Kreislaufverhältnisse bei Wiederbelebung durch Veränderung des intrapulmonalen Druckes. Ztschr. f. d. ges. exper. Med., 1932, 82, 255.
8. Bruns, O., Die weitere Ausgestaltung der Unterdruckatmung für die Behandlung der Kreislauf und Atmungsstörungen. Med. Klinik, 1913, 42, 1714.
9. Böger, A., Cobet, R., and Stepp, W., Experimentelle Untersuchungen zur Frage der Steigerung der Herzleistung durch Unterdruckatmung. Arch. f. exper. Path. u. Pharm., 1931, 160, 329.
10. Warner, E. C., and Hambley, W. D., An investigation into the physiological basis of the U-tube manometer test. The physical fitness of men assessed by various methods. Guy's Hosp. Rep., 1925, 75, 286 and 388.
11. Flack, M., Tests for flying efficiency and flying strain. Med. Res. Council. Special Report, 1920, 53, 95.
12. Böger, A., Über die Einwirkung der Pressatmung auf den Kreislauf. Arch. f. exper. Path. u. Pharm., 1932, 166, 101
13. Bürger, M., Bürger, H., and Petersen, P. F., Die Pressdruckprobe als Herzleistungsprüfung. Arbeitsphysiol., 1929, 1, 614.
14. Petersen, P. F., Zur Frage des plötzlichen Ertrinkungstodes. Ztschr. f. d. ges. exper. Med., 1928, 61, 390.
15. Barach, A. L., Eckman, M., Ginsburg, E., Rumsey, C. C., Jr., Kor, I., Eckman, I., and Besson, G., Studies on positive pressure respiration. J. Aviation Med., 1946, 17, 290.
16. Kuhn, E., Eine Lungensaugmaske zur Erzeugung von Stauungshyperämie in den Lungen. Deut. Med. Woch., 1906, 32, 1486.
17. Kuhn, E., Weitere Erfahrungen mit der Hyperämiebehandlung der Lungen vermittels der Lungen Saugmaske. Munch. Med. Woch., 1907, 54, 782.
18. Bruns, O., Die kunstliche Luftdruckniedrigung über den Lungen. Munch. Med. Woch., 1910, 57, 2169.
19. Stigler, R., Die Kraft unserer Inspirationsmuskulatur. Pflüg. Arch. für ges. Physiol., 1911, 139, 234.
20. Rahn, H., Otis, A. B., Chadwick, L. E., and Fenn, W. O., The pressure-volume diagram of the thorax and lung. Am. J. Physiol., 1946, 146, 161.
21. Holt, J. P., The collapse factor in the measurement of venous pressure. Am. J. Physiol., 1941, 134, 292.
22. Holt, J. P., The effect of positive and negative intrathoracic pressure on peripheral venous pressure in man. Am. J. Physiol., 1943, 139, 208.
23. Otis, A. B., Rahn, H., and Fenn, W. O., Venous pressure changes associated with positive intrapulmonary pressures. Their relationship to the distensibility of the lung. Am. J. Physiol., 1946, 146, 307.
24. Otis, A. B., Rahn, H., Brontman, M., Mullins, L. J., and Fenn, W. O., Ballistocardiographic study of changes in cardiac output due to respiration. J. Clin. Invest., 1946, 25, 413.
25. Hamilton, W. F., Woodbury, R. A., and Harper, Jr., H. T., Physiologic relationships between intrathoracic, intraspinal and arterial pressures. J. A. M. A., 1936, 107, 853.
26. Bürger, M., Über die Bedeutung des intrapulmonalen Drucks für den Mechanismus des Kollapses bei acuten Anstrengungen. Zeit. exper. Med., 1925, 52, 321.
27. Heinbecker, P., The mechanism of the respiratory waves in systemic arterial blood pressure. Am. J. Physiol., 1927, 81, 170.
28. Laux, L., Über den Anteil der Kapazitäts und der Widerstandsänderungen der Lungenbahn an den respiratorischen Schwankungen der Stromstärke. Arch. f. d. ges. Physiol., 1930, 224, 110.
29. Shuler, R. H., Ensor, C., Gunning, R. E., Moss, W. G., and Johnson, V., The differential effects of respiration on the left and right ventricles. Am. J. Physiol., 1942, 137, 620.
30. Daly, deBurgh I., Resistance of the pulmonary vascular bed. J. Physiol., 1930, 69, 239.
31. Herrick, J. F., Code, C. F., MacLean, A. R., and Mann, F. C., Vascular changes associated with short periods of raised intrapulmonary pressures. Fed. Proc., 1942, 1, 39.
32. Bruns, O., Ziele und Erfolge der Behandlung von Kreislaufstörungen mit Unterdruckatmung. Deut. Med. Woch., 1911, 37, 2214.

STUDIES ON GANGRENE FOLLOWING COLD INJURY: VI. CAPILLARY BLOOD FLOW AFTER COLD INJURY, THE EFFECTS OF RAPID WARMING, AND SYMPATHETIC BLOCK[1]

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Indirect evidence, based upon measurements of skin temperature and observations of skin color in the feet and ears of rabbits subjected to severe cold injury, indicates that complete arrest of blood flow does not occur until more than 50 hours after injury (1). Tests of the local circulation in cold-injured regions with intravenously injected fluorescein show that the exchange of this dye between blood and interstitial fluid is impaired during the interval when the minute volume blood flow is greater than that in comparable uninjured regions (2, 3). Early arrest of blood flow in regions injured by cold has been variously ascribed to "conglutination" of red-cells (4), to capillary stasis (5), and to intravascular clotting (2). While all of the above phenomena may be observed in frostbitten tissues, none has provided an explanation of the nature of local changes in blood flow adequate to account for the maintenance of high peripheral tissue temperature at a time following cold injury when exchanges of oxygen, nutrients, and metabolites appear to be impaired.

This report presents the results of microscopic study of blood flow in the small vessels of the ears of rabbits during the first hour after freezing. Blood flow was also studied in the ears of animals which were treated by rapid thawing of the ear in warm water and by procaine block of the stellate ganglion on the injured side.

METHODS

Cold injury was produced by immersing the distal one-half to one-third of the ear for 60 seconds in a mixture of water, ethylene glycol, and alcohol cooled to $-55°$ C. with solid carbon dioxide. Details of this method for the production of controlled cold injury and the characteristic effects on the tissues are given elsewhere (6).

Both normal and frostbitten ears of a uniform strain of New Zealand white rabbits were observed through a binocular dissecting microscope having 9X oculars and 4.8X objectives. The light sources was a 100-watt Spencer microscope lamp from which the light was conducted to the ear by means of a polished rod of ¾-inch methyl methacrylate (Lucite) 2 feet long. The rod was tapered to a cone having a rounded tip 7 mm. in diameter. The distal 8 inches of rod were bent into a curve which presented the brilliantly illuminated tip at a right angle to the original path of light. With the rod in an adjustable clamp, its flattened end in contact with the blue glass of the microscope lamp, the ear was placed under the objectives of the microscope in direct contact with the methacrylate rod. Liquid petrolatum was applied to the shaved ear in order to clear the keratinized epithelium. Photographic recording of the changes observed proved to be unsatisfactory because of the thickness of tissue and the changing optical plane.

OBSERVATIONS

Blood flow in the capillaries of the normal ear. Movement of blood could not be seen in the larger vessels. In the smaller arterioles and venules, rapid flow was easily made out. Numerous channels affording relatively direct communication between arterioles and venules were seen. These channels were usually of smaller diameter than either their supplying arterioles or the receiving venules, and many of them were small enough to force the corpuscles to traverse them in single file. Reversal of flow was not observed in these vessels, and the flow was uniformly rapid. The relatively few true capillaries which were open at any one time could be identified as richly anastomosing branches containing corpuscles either singly or in small clumps interrupted by clear areas. Often the location of true capillaries could be identified only when an occasional corpuscle was seen to wander off through an area apparently free of vessels and then finally to enter a collecting capillary and venule.

Hyperemia induced by the application of heat caused opening of vessels of all types, and many

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

regions, which had been entirely free of visible channels, were seen to be richly supplied with capillaries.

Blood flow in the capillaries of the ear after frostbite. Observations of blood flow were made on the normal and the frostbitten regions of ears of 15 rabbits. The changes which were observed in the local circulation were consistently reproducible in terms of their time relations and nature. The following description applies to the sequence of changes observed in the blood flow within the frostbitten area after the distal half of the ear had been immersed in the freezing mixture at − 55° C. for 1 minute and then allowed to thaw in air at room temperature without additional treatment. Blood flow began in the largest channels of the frostbitten portion of the ear within 3 minutes and before the ear was completely thawed. Flow in those channels was rapid and at that early stage only a limited number of capillaries in the immediate vicinity of large vessels became filled with blood. At the completion of thawing, the ears became markedly hyperemic. Progressive opening of capillaries was observed, and brisk blood flow could be detected for a few minutes after the capillaries became visible, but over a period of about 10 minutes slowing and final cessation of flow occurred. The capillaries contained packed red blood cells interrupted by clear regions along the length of the vessel. Within the interval, 10 to 20 minutes after frostbite, these vessels became completely filled with cells, their course was grossly distorted, and the walls were roughened and irregular.

The period of onset of stasis was accompanied by increasing edema. A homogeneous brownish substance began to accumulate in the areas adjacent to the capillaries and was found in largest amounts near the larger blood vessels in which flow persisted longest. Although no actual movement of blood could be observed, sluggish flow could be demonstrated by observing the refilling of vessels after they had been emptied of blood by pressure upon an overlying coverglass. Such persistence of flow was demonstrated in the larger arterioles and venules and also in some small, relatively straight vessels. The latter were bright pink in color and had smooth walls; their diameter was slightly smaller than that of the distended capillaries which contained packed red cells.

Vessels in the area proximal to the frostbitten region were widely dilated for the first 20 to 50 minutes after thawing. Usually within 2 hours these vessels became markedly reduced in diameter. The central artery and its branches were more noticeably affected than the veins, and there appeared to be a region of especially marked narrowing of the artery at the junction of the normal with the injured area. At the same time it was noticed that the large vessels within the frostbitten part of the ear remained widely dilated.

Study of the circulation after frostbite with the aid of intravenous injections of India ink. Intravenous injections of particulate carbon in the form of India ink were used to assist in the visualization of blood vessels in both normal and frostbitten ears. The ink was administered in doses of 5 ml. of 50 per cent India ink in saline both immediately before frostbite and at various intervals from 2.5 minutes to 49 minutes after the ear was removed from the freezing mixture. The circulation in the ears of 10 animals was observed after the injection of carbon.

By the selection of the appropriate time interval for the injection of carbon in relation to the time of frostbite it was possible to demonstrate the following: (*a*) carbon particles present in the circulating blood either just before frostbite or within 6 minutes afterward entered the capillaries of the frostbitten region; (*b*) varying amounts of carbon became trapped in the capillaries of both the normal and the frostbitten parts of the ear; (*c*) carbon trapped in capillaries of the normal part of the ear was removed by the increased blood flow when hyperemia was induced by heat, but particles caught in the dilated capillaries of the frostbitten region during the period of stasis could not be removed by this means; (*d*) up to 6 minutes after frostbite, more carbon was trapped in the capillaries of the frostbitten region when carbon injections were made late in this 6-minute interval than after pre-frostbite injection or very early post-frostbite injection of ink; (*e*) when the injection of ink was delayed as long as 20 minutes after frostbite, only occasional particles of carbon could be seen in the vessels of the frostbitten region,—when these did appear, they were seen only in the relatively large channels and not in the true capillaries.

Thus, the interval during which the entrance

and trapping of carbon particles could occur was brief and was terminated by stasis. The channels which did remain open contained flowing blood, were relatively large in diameter, and hence probably permitted any carbon which entered them to be carried on through.

The junction of the normal part of the ear with the frostbitten region exhibited some differences from both regions distal and proximal to it. When India ink was injected after stasis had become complete in the true capillaries, *i.e.*, more than 20 minutes after frostbite, carbon accumulated and became trapped at the junction when none or very little could be found in either the frostbitten region or the normal part of the ear. This observation suggests that the stasis which occurred here was relatively late in appearing, since it was not sufficiently advanced to prevent the entrance of carbon particles but later did progress to the point where large amounts of ink were trapped.

While no positive explanation of the above observation is justified on the basis of our present information, it is suggested that the tissues of the junction may have been less severely injured by reason of their failure to reach temperatures as low as those in the more distal portion of the ear. Since swelling progressed from the injured region toward the base of the ear, distortion and occlusion of capillaries may account for late appearance of stasis in the region of the junction even though the direct injury by cold was not severe enough alone to induce stasis.

Local blood flow in frostbitten ears after rapid thawing in warm water. Frostbite produced in the ears of rabbits by immersing the part in the freezing mixture at − 55° C. for 1 minute resulted invariably in the development of gangrene and the ultimate loss of all of the ear up to the line of immersion, if the ears were allowed to thaw (in air at 25° C.) without treatment. In confirmation of the work of Ariev (7), we have found that the same exposure to cold followed by immediate warming of the ear in water at + 42° C. for 2 minutes prevents the development of gangrene and loss of the injured tissue (8). In spite of the favorable end result of rapid thawing, the tissues underwent a series of changes which were grossly similar to the pregangrenous changes in the frozen, untreated ears. The stages of hyperemia, swelling and later exudation of serous fluid from the surface of the injured part of the ears were, if anything, more marked than in the untreated ears, but in spite of these alarming manifestations, only the surface epithelium was lost.

Observations were carried out in the manner described previously, and injections of India ink were used to test for the appearance of stasis. The ears of 3 animals were studied after subjecting both ears to frostbite (1 minute at − 55° C.) and thawing one of them rapidly in warm water. The injections of ink were made at 20 to 25 minutes after frostbite and were thus administered during the period when capillary stasis is complete in similarly injured, untreated ears.

Before the intravenous injection of carbon, the changes of blood flow observed in the rapidly warmed ear were not strikingly different from those in the contralateral, frostbitten but untreated ear. Refilling of vessels emptied by external pressure persisted longer in the treated ear and larger amounts of brown extravascular material appeared. When particulate carbon was introduced into the blood stream, it was seen to enter the treated ear more readily than the untreated one in 2 out of 3 rabbits studied. In the third, the amounts of carbon were about the same in both ears (not more than 3 to 4 particles per field). For 1 or 2 hours after the injection of ink, the particles were either observed to move spontaneously in the vessels of the treated ear or could be made to move by mechanical manipulation. In one experiment, hyperemia was induced in the treated ear by infiltrating the tissues about the base of the ear with 1 per cent procaine 77 minutes after the injection of ink. The increased blood flow removed some of the carbon which previously had been trapped. Hyperemia induced by this means or by warming was shown to be ineffective in removing trapped carbon from frostbitten ears which had not been treated by rapid thawing.

The observations showed that rapid thawing of frostbitten ears had the effect of delaying complete stasis in the true capillaries. When stasis did develop, it coincided in time with the period of maximal swelling (approximately 2 hours after injury) and with the reduced blood flow brought about by the constriction of arterial supply in the uninjured portion of the ear. Thus, when perfusion pressure within the capillaries was dimin-

ished, and distortion of the tissues by the presence of large amounts of extravascular fluid occurred, cessation of flow in the true capillaries was an accompanying feature. However, flow of blood in the true capillaries could be made to proceed by changing the pressure relationships within those vessels either by increasing the flow of blood through the injured tissue or by mechanical pressure applied externally and released.

The local circulation in frostbitten ears following procaine block of the stellate ganglion. In order to determine whether the effects observed after rapid warming of frostbitten ears were the result of the changes in blood supply alone or whether some other effect of rapid warming was responsible for the delay of capillary stasis, 6 rabbits were subjected to frostbite of the distal half of both ears in the same manner as those described in the previous sections, and as soon as possible after the ears were removed from the freezing mixture, 1 to 2 ml. of 2 per cent procaine with epinephrine 1:30,000 were injected into the region of the stellate ganglion on one side. All of the animals in this group demonstrated satisfactory paralysis of the vasomotor supply of the ear as judged from the presence of the more readily palpable pulse in the central artery and skin temperatures 4° to 6° C. higher on the blocked side.

When carbon was injected into the blood stream within 5 minutes after frostbite, large amounts of it were observed in the capillaries of the frostbitten, untreated ear, but relatively little of it was found in the ear subjected to vasomotor paralysis. Stasis in the untreated ear resulted in the immobilization of large amounts of carbon, but in the capillaries of the ear on the side blocked by procaine, blood flow and movement of carbon particles persisted for more than 2 hours. Two hours and 10 minutes after the injection of India ink some stasis was noted in capillaries close to the surface; these vessels could not be emptied by external pressure, but in spite of this late stasis there was no trapping of carbon except at the junction of normal and frostbitten and normal tissue. In this case failure of trapping may have been due to removal of carbon from the circulation before stasis developed. When the injection of carbon was delayed until 22 minutes after frostbite, the usual exclusion of carbon particles from the capillaries of the injured but untreated ear was observed. In the ear which was treated by the production of vasomotor paralysis, the capillaries were seen to contain moving carbon particles, but within an additional 30 minutes stasis occurred with immobilization of carbon granules. In this animal the duration of increased blood flow was rather brief, and both ears were cool and about the same temperature by the time that stasis was observed in the treated ear. The vessels did not refill after being emptied by external pressure.

These observations indicate that the increased blood flow which followed stellate ganglion block was associated with a delay of stasis in the true capillaries of frostbitten ears. While stasis was not postponed for a period as long as that following rapid thawing of the ear in warm water, it was qualitatively similar. This suggests that at least a part of the improvement in capillary blood flow noted after rapid thawing may be the result of increased intravascular pressure. However, that rapid thawing produced other changes in the response of the tissues was evident from the difference in final result: preservation of the frostbitten region after rapid thawing as compared with merely the delay for 24 hours of the wet and dry stages of gangrene and loss of the injured portions of the ear, which was the uniform experience after the use of procaine block.

Lymph flow and the spread of colloidal dye in frostbitten skin. Glenn, Gilbert and Drinker (9) reported an augmented lymph flow for at least 2 hours following severe hot water burns of the foot in dogs. Massive swelling was a constant feature of the response to injury in their experiments. The high rate of lymph flow points to a dynamic rather than a static alteration of fluid distribution in the injured tissues. In view of the many similarities between the vascular disturbance in burns and frostbite (10) the demonstration of augmented lymph flow in the frostbitten extremities of rabbits might throw some light on the nature of fluid exchanges following severe injury by cold. It has been pointed out that the edematous tissues after frostbite are to some extent organized (1), in that only limited quantities of edema fluid flow from the incised swelling. Organization of this fluid either in the form of a fibrin clot or in a gel stabilized by hyaluronic acid (11) might offer some mechanical barrier to free movement of fluid and plasma protein in the extravascular compart-

ment and also hinder the movement of fluid into capillaries even though adequate filtration gradients existed.

A small number of experiments designed to demonstrate lymph flow by the use of the dye Patent Blue V as employed by McMaster (12) and the state of the interstitial fluid by the spread of T–1824 by the method of McMaster and Parsons (13) were carried out on the frostbitten and normal ears of rabbits. Two rabbits were used for the studies with Patent Blue V. One ear of each animal was frostbitten by immersion in the freezing mixture for 1 minute at − 55° C. The contralateral ear of each animal was used for control observations. Patent Blue V (0.01 ml. of an 11 per cent solution in water) was injected intradermally through a G–26 hypodermic needle. In one animal, the dye was introduced into both ears midway between the central artery and the marginal vein 4.5 hours after frostbite. In the frostbitten ear diffuse, blue streamers were seen to extend proximally within 5 seconds. After 10 seconds, a poorly defined blue band extended from the point of injection to the marginal vein. These bands blended into the neighboring skin areas with no clear, fine lines. The color became diffuse and faded completely within a few minutes. In the normal ear of the same animal, narrow, sharply defined blue lines radiated from the point of injection. Within 60 seconds after injection, a small blue streamer reached the marginal vein; and, after 4 to 5 minutes, fine blue lines extended proximally along the central artery.

A second rabbit was subjected to the same degree of injury as the first, but the dye was injected 30 minutes after frostbite. Rapid and diffuse spread of dye in the frostbitten ear toward the central artery and laterally toward the marginal vein was observed. Two hours after injection of dye, diffuse, blue streamers extended proximally along the central artery and near the marginal vein. After 5.5 hours all dye had disappeared from the frostbitten ear. In the normal ear, fine lines extended proximally along the central artery, but these moved at a much slower rate than those in the frostbitten ear. After 2 hours no streamers were seen. A large amount of dye remained at the original site of injection at the end of 5.5 hours.

These observations indicate that lymph flow in the frostbitten ears of rabbits persisted after injury and that it was more rapid than that observed in normal ears.

The spread of T–1824 in normal and frostbitten skin of rabbits' ears was studied after injection of 0.01 ml. of dye (1 per cent T–1824 in isotonic sodium chloride solution) intradermally into both ears after one of them had been frostbitten in the usual manner. The size of the spot of dye was measured at intervals over periods of 17 to 21 hours. Seven rabbits were studied in the manner described. The results in all 7 animals were essentially similar. Spread of dye occurred in both normal and frostbitten ears but was more extensive in the frostbitten ears than in the normal ones. A single example will suffice to indicate the relative magnitude and qualitative aspects of changes observed. The size of the spot of dye immediately after injection was 4 × 4 mm. in each ear. At 170 minutes after injection of dye the spot had increased to 6 × 11 mm. in the normal ear while that in the frostbitten ear was 15 × 60 mm. After 21 hours the dimensions were 11 × 13 mm. in the normal ear while no dye was detectable in the frostbitten ear at this time.

The results of similar measurements on an animal in which the frostbitten ear was thawed rapidly in warm water (3 minutes at + 42° C.) did not differ strikingly from those obtained on untreated, frostbitten ears. A series of 5 animals studied after marked edema had been induced in one ear by wetting it with xylene showed differences between the edematous and the normal ears which were similar to those observed when edema was produced by frostbite.

As judged from the observations made with intradermally injected dye in frostbitten ears, there appears to be no evidence of physical organization of the edema in such a way as to impede the spread of colloidally dispersed material within it.

COMMENT

Direct observations of blood flow changes in the minute vessels of normal and frostbitten ears of rabbits, both with and without intravascular India ink injections, are in substantial agreement with those reported by Tittel (14) and by Rotnes and Kreyberg (15). The following points are brought out:

(*1*) Cold injury leads to stasis in the true capillaries in proportion to the severity of injury as it is determined by time of exposure and temperature.

(*2*) Following a standard cold injury of 1 minute immersion of the distal half of the ear in the freezing mixture at − 55° C.:

(*a*) Blood flows into all vessels of the ear in the early period after thawing.

(*b*) Stasis appears first in the true capillaries, begins with the return of blood flow, and is complete in the true capillaries within 10 minutes after the first reappearance of blood in the injured region.

(*c*) Stasis is accompanied by loss of fluid from the true capillaries into the surrounding tissues and by dense packing of erythrocytes within the capillaries.

(*d*) Carbon particles (India ink), injected into the blood stream before stasis is complete, become trapped in the true capillaries during the development of stasis, but if the injection of carbon is delayed until after stasis is complete, none of it enters and none is trapped.

(*e*) Blood flow in the cold-injured region, after stasis has closed the true capillaries, occurs in arteriovenous anastomoses and in arteriolar-venular capillaries, described as "thoroughfare channels" by Chambers and Zweifach (16).

It is evident from the above summary that during the hyperemic phase after injury the total cross sectional area of the vascular bed between the arterioles and venules within the frostbitten region becomes sharply reduced to include only a-v anastomoses and arteriolar-venular capillaries. This response to injury by cold has been noted by other investigators (17 to 22). An important consequence of such a local redistribution of blood flow in the presence of a total increase in minute volume flow, as indicated by the changes of skin temperature, would be a marked rise in effective filtration pressure. Thus, in spite of the fact that the true capillaries may lose large amounts of fluid up to the time that stasis is complete, the later loss of fluid into the extravascular compartment must occur mainly as the result of the high filtration pressure within the "thoroughfare channels." The measurement of changes in volume and subcutaneous tissue pressure in frostbitten feet of rabbits (23) showed that maximum volume is reached in about 2 hours after injury and that the pressure of fluid in the interstitial compartment reaches its maximum of 25 to 30 cm. of water at about the same time. In the period between thawing and the attainment of maximum swelling a new filtration equilibrium is established at high levels of capillary pressure, interstitial fluid pressure, and rate of fluid exchange. That fluid continues to be lost from the blood in the injured regions at rates which overwhelm the local routes of removal is illustrated by 2 observations: (*a*) the movement of edema fluid in the subcutaneous tissues far into the uninjured regions from the area injured by cold (23); and (*b*) the rapid lymph flow from the injured region as demonstrated with the aid of dyes.

The "flux" of plasma ultrafiltrate in normal tissues has been described by Zweifach (24) as filtration from the plasma in the capillaries with high internal pressure (the arteriolar-venular capillaries) and the re-entry of plasma ultrafiltrate into the blood stream by seepage into true capillaries, especially in the region close to their junction with an a-v capillary as it enters a collecting venule. In frostbitten ears of rabbits, the closure of true capillaries and the rapid inflow of blood during the hyperemic stage favor the vigorous formation of plasma ultrafiltrate and perhaps even the loss of some protein through the walls of a-v capillaries; but the stasis, effective in diverting inflowing blood into "thoroughfare channels" at high pressure, at the same time renders the true capillaries useless as routes of re-entry of fluid into the blood stream. In addition to closure by primary stasis, obstruction of some capillaries may be accomplished as a result of the spatial distortion and kinking which occur during swelling.

The effect of closure of true capillaries upon exchanges between the blood and the extravascular compartment is that of sharply limiting the available surface across which diffusion may take place and at the same time increasing the rate of filtration. Danielli and Stock (25) and Zweifach (24) have pointed out the separate nature of exchanges by diffusion and exchanges which occur largely by filtration. Resting tissues characteristically depend upon the flux of plasma ultrafiltrate; minute volume blood flow is relatively small and only a few true capillaries are open at a time. Active tissues depend upon a considerable increase in the area

available for diffusion as well as upon increased filtration. Thus, in active tissues the minute volume blood flow is large, many true capillaries are open, and an appreciable volume of filtrate is formed. In frostbitten tissues an anomalous and probably harmful situation exists: blood flow and local temperature are high at a time when the total area available for diffusion exchange is reduced. In spite of the high temperatures, which might be expected to increase the metabolism of tissues in the injured region, measurements of arterio-venous oxygen differences in the blood perfusing these tissues indicate that the rate of oxygen consumption is extremely low (23).

SUMMARY

1. Paralysis of vasomotor activity, both tonic contraction and phasic responses, was produced in the injured regions when the distal halves of rabbits' ears were exposed to $-55°$ C. for 1 minute in a liquid freezing mixture. Vasomotor activity was preserved in vessels proximal to the line of immersion.

2. After thawing of the frozen ears in air at 25° C., blood flow was re-established and all vessels became markedly dilated. Stasis began in the true capillaries with the return of blood flow and was complete in 10 minutes.

3. Particulate carbon, injected intravenously, became trapped in the true capillaries when injection was made within the first 10 minutes but failed to enter true capillaries when injection was delayed for longer intervals after injury.

4. Blood flow persisted in arterio-venous anastomoses and in arteriolar-venular capillaries for about 24 hours but declined virtually to zero over the ensuing 24 to 30 hours.

5. Rapid thawing of frozen ears in warm water (1 to 3 minutes at $+42°$ C.) delayed the development of stasis in the true capillaries until maximal swelling was reached at about 2 hours after injury.

6. Procaine block of the stellate ganglion augmented the hyperemia after injury and delayed the onset of stasis for 50 to 60 minutes.

7. Persistence of rapid lymph flow and rapid formation of interstitial fluid in frostbitten ears up to 21 hours after injury was demonstrated with the aid of intradermal injections of the dyes, Patent Blue V and T-1824.

BIBLIOGRAPHY

1. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. II. General course of events in rabbit feet and ears following untreated cold injury. J. Clin. Invest., 1947, 26, 236.
2. Lange, Kurt, and Boyd, L. J., The functional pathology of experimental frostbite and the prevention of subsequent gangrene. Surg., Gynec. & Obstet., 1945, 80, 346.
3. Crismon, J. M., and Fuhrman, F. A., Studies on gangrene following cold injury. V. The use of fluorescein as an indicator of local blood flow: fluorescein tests in experimental frostbite. J. Clin. Invest., 1947, 26, 268.
4. Kreyberg, L., Some notes and considerations regarding injuries from cold. 1945, 108th (U. S.) General Hospital.
5. Bigelow, W. G., Modern conception and treatment of frostbite. Canad. M. A. J., 1942, 47, 529.
6. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. I. A method for producing gangrene by means of controlled injury by cold. J. Clin. Invest., 1947, 26, 229.
7. Ariev, T. Y., Slow *vs.* rapid warming of frozen extremities. Vestn. Khirur., 1939, 57, 527.
8. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. VII. Treatment of cold injury by means of immediate rapid warming. J. Clin. Invest., 1947, 26, 476.
9. Glenn, Wm. W. L., Gilbert, Helen H., and Drinker, C. K., The treatment of burns by the closed-plaster method, with certain physiological considerations implicit in the success of this technique. J. Clin. Invest., 1943, 22, 609.
10. Harkins, H. N., The Treatment of Burns. C. C. Thomas, Springfield, Ill., 1942.
11. Meyer, K., and Chaffee, E., The muco-polysaccharides of skin. J. Biol. Chem., 1941, 138, 491.
12. McMaster, P. D., Lymphatic participation in cutaneous phenomena. Harvey Lectures, 1942, 37, 227.
13. McMaster, P. D., and Parsons, R. J., The method of interstitial spread of vital dyes and the state of the fluid in the intradermal tissue. J. Exper. Med., 1939, 69, 247.
14. Tittel, S., Über die Reaktionsweise des Gefässsystems bei lokaler Erfrierung. Ztschr. f. d. ges. exper. Med., 1944, 113, 698.
15. Rotnes, P. L., and Kreyberg, L., Eine Methode zum experimentellen Nachweis von Stase mittels spezieller Präparate. Acta path. et. microbiol. Scand., 1932, Suppl. 11, 162.
16. Chambers, R., and Zweifach, B. W., Topography and function of the mesenteric capillary circulation. Am. J. Anat., 1944, 75, 173.
17. Grant, R. T., and Bland, E. F., Observations on arteriovenous anastomoses in human skin and in the bird's foot with special reference to the reaction to cold. Heart, 1929–31, 15, 385.

18. Grant, R. T., Observations on direct communications between arteries and veins in the rabbit's ear. Heart, 1929–31, **15**, 281.

19. Jochim, K. E., and Hertzman, A. B., Vascular reactions to cold related to the early stages of trench foot. Proc. Fed. Am. Soc. Exper. Biol., 1944, **3**, 22.

20. Hertzman, A. B., and Roth, L. W., The reactions of the digital artery and minute pad arteries to local cold. Am. J. Physiol., 1942, **136**, 680.

21. Theis, F. V., Frostbite of extremities: clinical and pathological considerations in its diagnosis and treatment. Arch. Phys. Therapy, 1940, **21**, 663.

22. Friedman, Nathan B., Capt. M. C., The pathology of trench foot. Am. J. Path., 1945, **21**, 387.

23. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. III. Edema following cold injury: its magnitude and the composition and source of edema fluid. J. Clin. Invest., 1947, **26**, 245.

24. Zweifach, B. W., The structural basis of permeability and other functions of blood capillaries. Cold Spring Harbor Symp. Quant. Biol., 1940, **8**, 216.

25. Danielli, J. F., and Stock, A., The structure and permeability of blood capillaries. Biol. Rev., 1944, **19**, 81.

STUDIES ON GANGRENE FOLLOWING COLD INJURY. VII. TREATMENT OF COLD-INJURY BY MEANS OF IMMEDIATE RAPID WARMING[1]

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It has long been considered that the immediate application of warmth to frostbitten extremities should be avoided. In 1939 the Russian investigator, Ariev (1), reviewed the experimental and clinical observations upon which the accepted procedure of slow, gradual warming is based. He concluded, on the basis of his experimental results (cited below), that rapid warming in cases of cold injury should be subjected to clinical trial. Two human cases were reported in which no ill effects were observed to follow rapid warming of the feet and legs.

The older literature, cited by Sonnenburg and Tschmarke (2), Ariev (1), and Harkins (3), contains many observations which have been interpreted to indicate the danger of rapid warming of frostbitten parts. These observations are, however, uncontrolled and in many cases open to doubt. Perhaps the outstanding example is Barron Larrey's description of cold injury among the soldiers of Napoleon's army during the retreat from Moscow in the winter of 1812–1813 (4):

> "Unfortunate was the fate of him, who, with his animal functions nearly annihilated, and his external sensibility almost destroyed by the cold, should suddenly enter too warm a room, or approach too nearly a large bivouac fire. The projecting parts of the body, grown insensible, or being frozen, and remote from the centre of circulation, were attacked with gangrene, which manifested itself at the same moment, and was developed with such rapidity, that its progress was perceptible by the eye, or else the individual was suddenly suffocated by a sort of turgescence, apparently invading the pulmonary and cerebral systems. He perished, as if in a state of asphyxia."

An experimental approach to the problem of slow *versus* rapid warming of frozen extremities was made in 1937 by Harkins and Harmon (5). Hind limbs of dogs were frozen with solid CO_2 and one leg was thawed at 42° C. for comparison with the other thawed at 2° to 12° C. One frostbitten ear of rabbits was thawed at 38° C. and the other at 2° C. In neither case was any essential difference reported between the rapidly and slowly thawed extremities, although the frostbitten legs were observed only for 18 hours. They reported that "if anything, the ears that were thawed in ice water showed a trace more gangrene."

Ariev (1) has reported the results of several series of experiments which were designed to compare the effect of slow and rapid warming of frozen ears and feet of rabbits. After freezing both ears of rabbits with ethyl chloride, one ear was warmed with hot compresses wet in water at 40° to 45° C. The frostbitten part of the slowly-warmed ear was ultimately lost in 5 animals while the rapidly-warmed ear showed only fibrosis in the distal part. Both feet of rabbits were frozen with ethyl chloride to the tibio-tarsal joint. One foot was then rapidly thawed by means of the application of compresses wet with water at 35° to 40° C. while the other was thawed in air. In the rapidly warmed foot, gangrene "only touched the most distal parts of the toes," while in the opposite foot "total gangrene spread over the entire region subjected to freezing."

The experiments reported here were carried out in order to determine the effectiveness of immediate rapid warming in preventing the occurrence of gangrene following controlled cold injury. The effect of rapid warming on local blood flow in frostbitten ears and feet following thawing has been examined by means of fluorescein and by measurement of skin temperature.

METHODS

The method used for the production of controlled cold injury has been described previously (6). In this series of experiments, rabbit ears were immersed at − 55° C. for 1, 1½ or 2 minutes; rabbit feet were immersed at − 55°

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

C. for 3 minutes and at − 15° C. for 60 minutes. The rapid warming was accomplished by immersion of the foot or ear, immediately after removal from the cold liquid, in a water bath at + 42° C. Ears were warmed for periods ranging from 15 to 120 seconds; feet were warmed for 2 minutes or 5 minutes. During immersion in the warm water the foot or ear was kept in constant motion to facilitate thawing.

The effectiveness of immediate rapid warming was evaluated on the basis of the extent of ultimate tissue loss, the time required for the development of wet and dry gangrene, and the extent of induration and scarring of the ear or foot several weeks after injury.

RESULTS

1. *Immediate rapid warming of rabbit ears following controlled cold injury.* The extent of tissue loss following frostbite of rabbit ears without treatment and with treatment in the form of immediate rapid warming is given in Table I. The entire distal part of the ear as far as the line to which it was immersed becomes gangrenous and is eventually lost when the ear is thawed in air at room temperature (untreated). After 60-second immersion at − 55° C. the procedure of immediate rapid warming resulted in saving the entire frostbitten part of the ear in 4 out of 6 animals, and in the retention of about 90 per cent of the injured region in 1 animal. With frostbite of longer duration (90- and 120-second immersion at − 55° C.) the procedure of rapid warming resulted in saving the entire area in 1 out of 4 animals and incomplete loss in 1 animal.

TABLE I

Tissue loss following severe cold injury in untreated animals and in animals treated by immediate rapid warming of ears

All ears frostbitten by immersion in liquid at − 55° C.

*Untreated control series**

Duration of exposure	Number animals	Extent of tissue loss
seconds		
60	11	Complete to level of immersion
90	24	Complete to level of immersion
120	2	Complete to level of immersion

Immediate rapid warming

Duration of exposure	Temp. of warm water	Duration of warming	Extent of tissue loss
seconds	*° C.*	*seconds*	
60	+41	15	None
60	+42	60	None
60	+42	120	None
60	+42	120	Complete to level of immersion
60	+42	120	Distal 1 cm.
60	+42	120	None
90	+42	30	None
90	+42	30	Distal 2 cm.
90	+42	120	Complete to level of immersion
120	+42	45	Complete to level of immersion

* These animals are the same as those included in paper I of this series (6).

In the treatment of frostbite of rabbit ears by immediate rapid warming, thawing of the frozen ear occurs 10 to 15 seconds after it is placed in warm water. Following removal from the warm bath the ear is intensely hyperemic and usually tends to be somewhat cyanotic. Edema is evident upon removal from the warm bath and exceeds that observed in untreated frostbitten ears during the first 24 hours. Blisters are frequently seen. Exudation of protein-containing fluid from the surface of the ear occurs after 24 to 48 hours and results in the accumulation of a crust of protein which covers the entire frostbitten part of the ear. The crust begins to crack and fall from the ear after 10 to 15 days, revealing a pink surface covered by a thin layer of epithelium. The ear beneath contains a large amount of fibrous tissue which shrinks during the next few weeks. After several weeks the ear is shrunken and thickened, but shows no loss of tissue as the results of injury. The appearance of frostbitten ears treated by means of rapid warming is shown in Figure 1. The course of events following frostbite of a rabbit ear, with and without rapid warming, is outlined in Table II. The times required for development of wet and dry gangrene were quite uniform in the animals treated by rapid warming and in controls.

2. *Immediate rapid warming of rabbit feet following controlled cold injury.* The extent of tissue loss following frostbite of rabbit feet without treatment and with treatment in the form of immediate rapid warming is given in Table III. In general, rabbit feet frostbitten for 3 minutes at − 55° C. and permitted to thaw in air at room temperature (untreated) develop gangrene over the entire injured area and are usually lost to the level of immersion (Table III; Figure 2B and 2C). Following untreated frostbite produced by immersion of rabbit feet for 60 minutes at − 15° C., gangrene develops over the whole of the injured

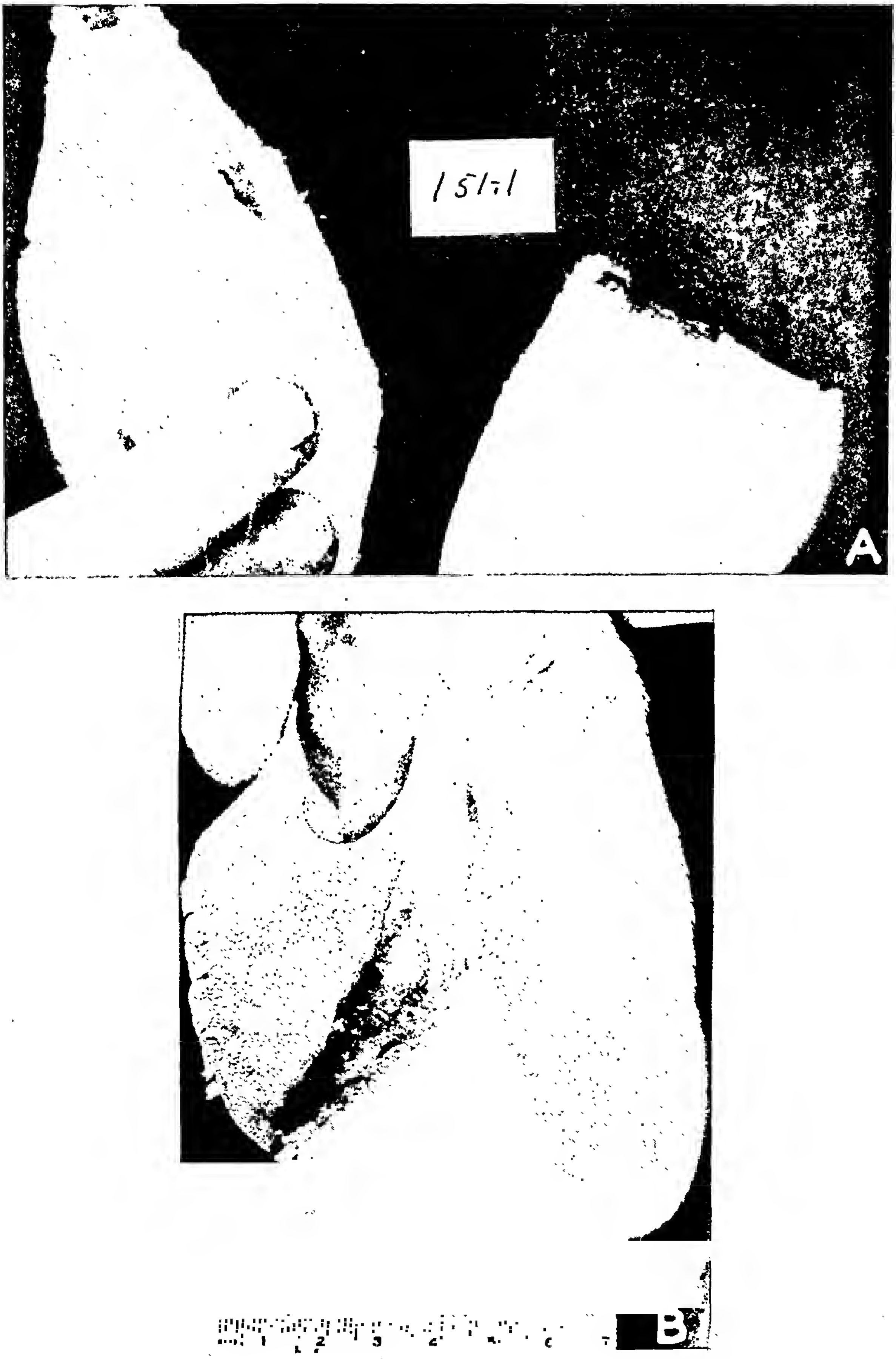

Fig. 1.

TABLE II

Effect of immediate rapid warming on the course of events following frostbite of rabbit ears

Time course of changes following severe cold injury of both ears of one rabbit. One ear treated by immediate rapid warming, while the other permitted to thaw at room temperature in air. Both ears injured by immersion for 60 seconds at −55° C. Right ear warmed in water at +42° C.

Days after injury	Right ear immediate rapid warming	Left ear untreated; thawed in air
1	Edematous. Warm. No blistering	Edematous. Cool. Moderate cyanosis. Large blister
2	Edematous. Warm	Edematous. Cool
3	Edematous. Warm	Weeping. Cool
5	Slight weeping. Warm	Weeping. Cool. Cyanotic. Wet gangrene on tip
6	Warm. Slight weeping. Encrusted with protein	Cold. Wet gangrene, but beginning to dry on tip
8	Encrusted with protein. Warm	Dry to level of frostbite
15	Crust peeling off. Frostbitten portion of ear intact	Spontaneous amputation at level to which immersed

area, and either the entire foot is lost to the level of immersion or a slender tongue of tissue on the plantar surface of the foot is retained.

Treatment of frostbite, following exposure at −55° C. for 3 minutes, by means of immediate rapid warming results in the survival of all of the injured region except the toes (Table III; Figure 2D). Rapid warming following exposure at −15° C. for 60 minutes reduces the extent of tissue loss so that the toes or parts of the toes only are lost (Table III; Figure 2E and 2F).

The course of events in rapidly warmed feet is similar to that in rapidly warmed ears. Thawing occurs after about 1 minute in the warm bath. Following removal from the warm water bath the foot usually appears cyanotic and very hyperemic. The edema is somewhat greater than in untreated feet. Exudation begins after about 2 days. Wet gangrene develops on the toes after 4 to 5 days, and drying follows within 1 to 2 days. The toes shrink, become mummified, and eventually separate.

TABLE III

Tissue loss of untreated frostbitten rabbit feet and of frostbitten rabbit feet treated by means of immediate rapid warming

One hind foot of rabbit immersed in cold liquid, at the indicated temperature, to the level of the tuberosity at the base of the fifth metatarsal

*Untreated control series**

Duration of immersion	Temp.	No. of animals	Extent of tissue loss
minutes	*° C.*		
3	−55	16	Complete to line of immersion (11).† Complete except plantar pad (4). All toes (1).
60	−15	5	Complete to line of immersion (2). Complete except plantar pad (2). None; *foot did not freeze* (1).

Immediate rapid warming

Temp. of warm water	Duration of warming	Extent of tissue loss
° C.	*minutes*	
	A. Feet exposed at −55° C. for 3 minutes	
+36	5	Complete except for 1 cm
+43	2	Toes only
+42	5	Parts of toes: II-3; IV-3**
+42	5	Toes only
+43	5	Toes only
+42	5	Toes only
+41	5	Toes only
	B. Feet exposed at −15° C. for 60 minutes	
+42	5	Parts of toes: II-2,3; III-3**
+42	5	Toes only
+42	5	Parts of toes: II-3; III-3**
+42	5	None
+42	5	Parts of toes: II-2,3; IV-2,3**

* These animals are the same as those included in paper I of this series (6).

** Roman numerals indicate digits. Arabic numbers indicate phalanges; 1 = proximal, 2 = middle, 3 = distal.

† Numbers in parentheses indicate number of animals.

rate. In some cases wet gangrene may develop on the dorsal surface of the foot, but this soon dries into a crust 1 to 2 mm. thick. This eschar separates after 10 to 14 days, leaving a pink, granulating surface which is soon covered by epithelium. The foot remains warm at all times in contrast to the fall in temperature which occurs in untreated feet after about 48 hours (7). In final appearance the foot is thickened, with moderate scarring especially on the dorsal surface (Figure 2, compare normal A with D, E, F).

FIG. 1. TREATMENT OF EXPERIMENTAL FROSTBITE BY MEANS OF IMMEDIATE RAPID WARMING.

A. Both ears exposed at −55° C. for 1 minute. The ear on the left was warmed for 2 minutes at 42° C. immediately after injury. The ear on the right received no treatment and was permitted to thaw in air at room temperature. Photographed 16 days after frostbite.

B. The ear on the left was exposed at −54° C. for 1 minute and then immediately warmed in water at 42° C. for 2 minutes. The ear on the right is normal. Photographed 21 days after injury.

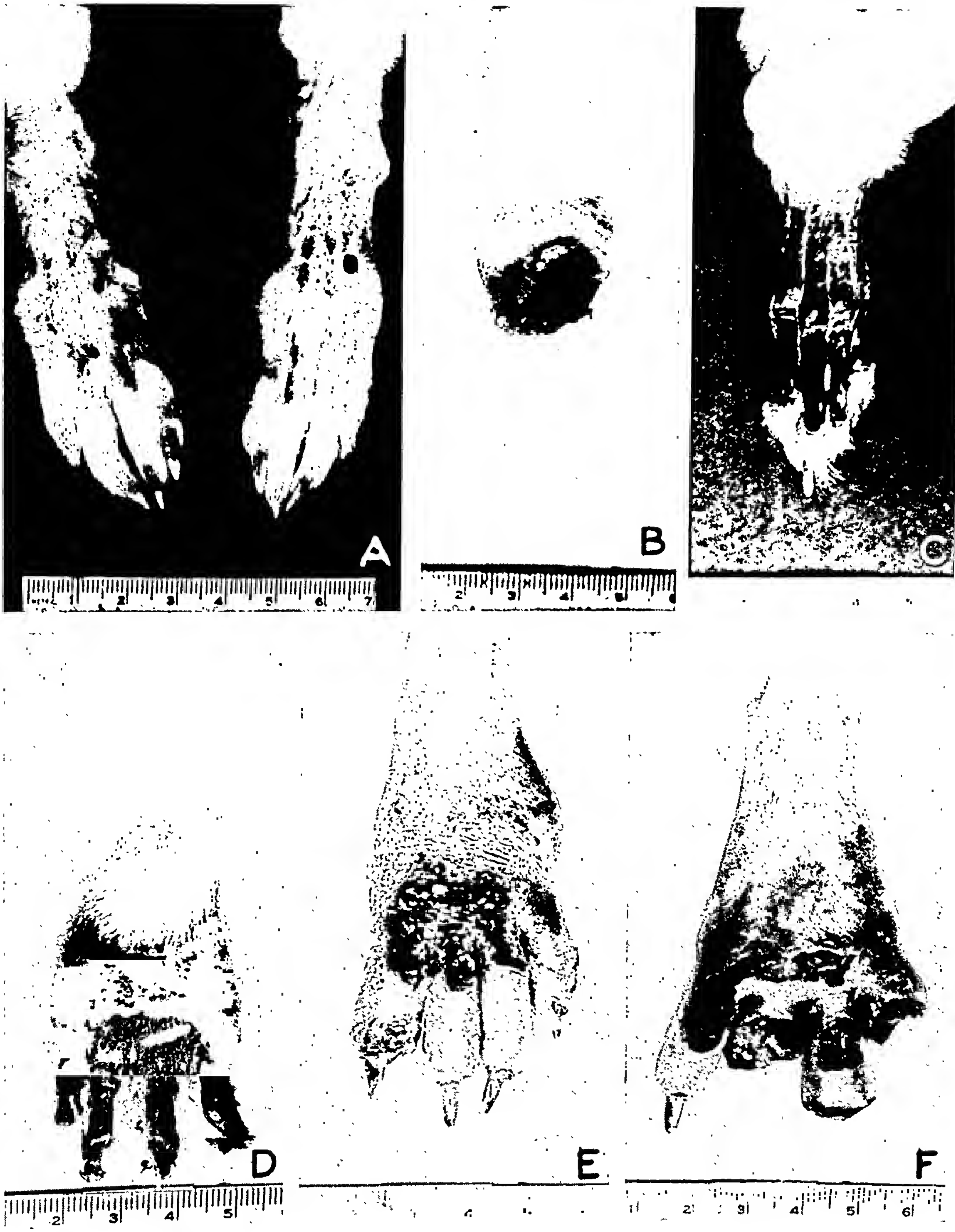

FIG. 2. TREATMENT OF FROSTBITE OF FEET BY MEANS OF RAPID WARMING

A. Normal rabbit feet clipped in preparation for frostbite.
B. Untreated frostbite 16 days after injury. Foot immersed at −55° C. for 3 minutes. Spontaneous amputation at the level of immersion occurred 14 days after frostbite.
C. Untreated frostbite 27 days after injury. Foot immersed at −55° C. for 3 minutes. Foot mummified to level of immersion but not separated.

After immediate rapid warming of feet exposed for 60 minutes at − 15° C., induration is more severe than in those feet frostbitten at − 55° C. for 3 minutes. Partial resolution of the induration occurs over a period of about 3 weeks, but sufficient periarticular fibrosis remains to cause spreading of the foot to 1.5 to 2 times its normal width. The fibrosis is particularly marked about the metatarsal-phalangeal joints (Figure 2E and 2F).

3. *Local status of the circulation in frostbitten ears and feet following immediate rapid warming.* Investigation of ears and feet of rabbits subjected to immediate rapid warming following cold injury has shown that certain important differences can be established between these rapidly warmed extremities and those which did not undergo treatment. Reference should be made to the previous papers in this series, cited below, for the details of the methods used.

a. Skin temperature. Skin temperature measurements of rabbit ears, which were immediately warmed for 2 minutes at + 42° C. following 60-second exposure at − 55° C., were made during the first few hours after injury and at intervals up to 5 days. Immersion of the frozen ear in warm water results in a rise in skin temperature to between 25° and 30° C. A further rise in temperature occurs during the next 30 minutes so that the maximum temperature does not differ markedly from that of untreated frostbitten ears at this time (7). During the first 5 to 6 hours after injury the skin temperature of the injured part of the rapidly warmed ears falls several degrees. The most striking differences in temperature between rapidly warmed ears and those thawed in air (control animals) are observed after 24 hours (Table IV). By this time the decline in temperature of the frostbitten untreated ear has become marked, while the frostbitten, rapidly warmed ear is at approximately the same temperature as the normal ears. Forty-eight hours after injury the temperature of the untreated frostbitten ear has fallen to that of the environment; the skin temperature of the rapidly warmed ears remains at 35° to 37° C. Skin temperature measurements of the ears of 3 untreated animals and 3 animals in which the frostbitten ears were rapidly warmed are given in Table IV. It is quite clear that blood flow through the frostbitten, rapidly warmed ears is maintained at a high level at all times during the period following injury.

TABLE IV

Effect of immediate rapid warming upon the skin temperature of frostbitten ears

Injured ear warmed at +42° C.				Injured ear thawed in air			
Animal number	Time after injury	Skin temperature: Injured ear	Skin temperature: Normal ear	Animal number	Time after injury	Skin temperature: Injured ear	Skin temperature: Normal ear
	hrs.	*° C.*			*hrs.*	*° C.*	
113	1 to 4	37.2	37.8	83	1 to 5	35.9	36.8
	20	35.5	35.0		29	24.4	37.3
	44	36.3	37.2		48	22.6	37.3
	68	35.2	36.0		95	24.3	34.4
					120	24.0	32.3
120	1 to 5	32.0	38.6				
	28	38.0	37.8	94	1 to 2	32.4	37.1
	47	34.3	34.5		27	35.8	37.2
	72	34.0	38.3		45	29.2	36.2
	96	37.3	37.5		118	24.5	37.9
121	1 to 3	31.8	33.6	93	1 to 6	35.1	37.7
	24	38.4	38.8		24	30.8	38.2
	49	35.9	37.1		31	28.5	34.9
	72	31.5	25.7		54	27.4	32.7
	119	35.7	37.2		72	27.2	37.2
					98	26.0	36.8
					150	24.3	37.6

b. Demonstrations with fluorescein of local circulation in frostbitten ears treated by rapid warming. The adequacy of the local circulation in untreated frostbitten ears and in frostbitten ears treated by immediate rapid warming has been examined by measuring the intensity of fluorescence under ultraviolet illumination in the injured region after the intravenous injection of fluorescein (8).

In 6 animals the distal half of the ear was frostbitten at − 55° C. for 60 to 90 seconds and im-

D. Frostbite treated by rapid warming. Photographed 26 days after immersion at − 55° C. for 3 minutes. Treated by immediate rapid warming in water at 42° C. for 5 minutes.
E. Frostbite treated by rapid warming. Photographed 16 days after immersion at − [illegible] C. for 60 minutes. Treated by immediate rapid warming in water at 42° C. for 5 minutes.
F. Frostbite treated by rapid warming. Photographed 14 days after immersion at − 15° [illegible] Treated by immediate rapid warming in water at 42° C. for 5 minutes.

mediately warmed in water at + 42° C. Sodium fluorescein, 75 mgm. per kgm., was then given intravenously. In some cases, a second injection of fluorescein was made after 24 or 72 hours. The fluorescence of the rapidly warmed ear was compared with that of the ear of another animal subjected to the same type of cold injury but permitted to thaw in air at + 23° C. The results in all animals were similar. When the fluorescein was given immediately after injury the entry into the rapidly warmed ear was more rapid than in the untreated ear and the maximum fluorescence was greater in the rapidly warmed ear. The differences became more marked when fluorescein was given at successively longer intervals after injury. Figure 3 shows the difference between a rapidly warmed ear (No. 18) and an ear thawed in air (No. 19) 18 hours after frostbite and 40 minutes after the administration of fluorescein. In the untreated ear, fluorescence is apparent only around the central artery and near the tip, in the region of large vessels. In the rapidly warmed ear fluorescence is fairly uniform.

Fluorescein injected 72 hours after injury usually fails entirely to enter the frostbitten untreated ear, while the rapidly warmed ear glows brightly.

In one animal both ears were frostbitten for 60 seconds at −55° C.; one ear was then rapidly warmed in water at + 42° C. Fluorescein was given immediately, and again after 48 hours. The curves showing the intensity of fluorescence as a function of time in both ears are given in Figure 4. After both injections, the fluorescence was more intense in the warmed ear. Forty-eight hours

Fig. 3. Fluorescence in Frostbitten Rabbit Ears after Fluorescein Administration

Fluorescence in rabbit ears 18 hours after frostbite and 40 minutes after the intravenous administration of 75 mgm. per kgm. sodium fluorescein. Ears frostbitten by immersion at − 56° C. for 90 seconds.
A. No. 18. Ear warmed in water at 42° C. for 2 minutes immediately after removal from freezing mixture.
B. No. 19. Ear permitted to thaw in room air (control).

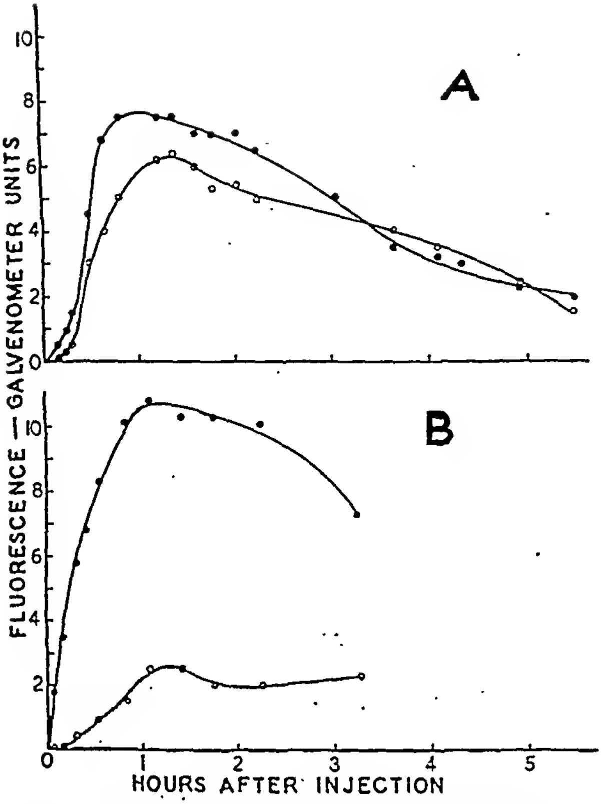

FIG. 4. EFFECT OF IMMEDIATE RAPID WARMING ON THE FLUORESCENCE OF FROSTBITTEN RABBIT EARS AFTER INTRAVENOUS ADMINISTRATION OF FLUORESCEIN

The results of a single experiment in which both ears of a rabbit were frostbitten by immersion at $-55°$ C. for 1 minute. Sodium fluorescein (75 mgm. per kgm.) given intravenously immediately after frostbite (A) and again 48 hours after frostbite (B).

O—O Ear thawed in air (control).

●—● Ear treated by immediate rapid warming by immersion in water at $+42°$ C. for 2 minutes.

after injury the maximum fluorescence of the rapidly warmed ear was 4.4 times that of the untreated ear.

The more rapid entry of fluorescein into rapidly warmed ears than into untreated frostbitten ears indicates that circumstances are present in the treated ears which permit the rapid establishment of equilibrium between the plasma and interstitial fluid in the ears. It is probable that this difference is a measure of the greater area through which fluorescein is able to diffuse into the rapidly warmed ear than the area through which it can pass in the untreated ear.

c. EFFECT OF RAPID WARMING UPON THE DEVELOPMENT OF STASIS AS DETERMINED WITH CARBON INJECTION. These data have been presented in detail previously as part of the description of local circulation following severe cold injury (9). Rapid warming of frostbitten ears had the effect of delaying the onset of complete stasis as determined by injection of carbon particles and direct observation of the trapping of carbon in the minute vessels of the ear. When stasis did develop in the rapidly warmed ears it coincided in time with the period of maximal swelling.

4. *Comparison of immediate rapid warming and cooling following severe cold injury.* Cooling of injured extremities has been recommended following cold injury of the trench foot or immersion foot type (10). In the treatment of frostbite, cooling in the form of cold baths (10° C.) and even rubbing with snow have often been recommended (11). From a theoretical point of view the maintenance of a low tissue temperature, and consequently of a low metabolic rate of the tissues, is desirable under circumstances in which blood flow is reduced.

In 2 animals the effect of cooling on the course of events following frostbite was studied for comparison with the results obtained by immediate warming. Both animals were frostbitten by immersion of one foot at $-54°$ C. for 3 minutes. The foot was then permitted to thaw in air at room temperature and was then cooled. The foot of one animal was suspended on a cradle inside a copper coil 3 inches in diameter, through which liquid at about $+5°$ C. was circulated. Air temperature inside the coil was maintained at 15° C. for 4 hours. The foot of the second animal was encased in rubber drainage tubing and cooled in cracked ice for 7 hours. In both animals the foot was lost to the level to which it had been immersed in the freezing mixture. The course of events leading to loss of the foot was similar to that in untreated animals.

DISCUSSION

Since any direct injury to the tissues resulting from the action of low temperature *per se* has already occurred by the time treatment can be instituted, any therapeutic measure, to be effective, must be directed toward prevention of the occurrence of the secondary changes which follow exposure to cold. S. S. Girgolav (12), director of a

bureau established in Leningrad under the Commissariat of Public Health, expresses a similar point of view in summarizing the Russian investigations: "From a clinical and pathologic standpoint, tissue necrosis in the body does not result immediately from the effect of cold, but appears as a secondary complication. . . . The course of necrosis is determined primarily by interference with or eventual complete stoppage in blood circulation."

The treatment of experimental frostbite by immediate rapid warming is an effective means of preventing the occurrence of the secondary changes following injury. The beneficial effects are probably not the result of shortening the length of time during which the part remains frozen. Thawing in water at + 36° C., rather than at + 42° C., was not effective in saving tissue even though the foot thawed within 2½ minutes after removal from the freezing bath. At 42° C. thawing requires approximately 2 minutes. In one experiment a foot frozen for 3 minutes at − 53° C. was then maintained in the frozen state at − 2° C. for 30 minutes. It was then thawed rapidly in water at + 42° C. for 5 minutes. The resulting tissue loss—toes only—was comparable to that obtained by rapid warming immediately after removal from the − 55° C. bath.

In confirmation of the work of Ariev (1) the immediate rapid warming of frostbitten feet and ears by immersion in water at + 42° C. has been shown to result in less extensive gangrene and smaller tissue loss than in untreated animals. Those ears and feet which were treated by this method underwent stages of hyperemia, massive edema, and exudation of serous fluid equal to, or exceeding, those of untreated animals. In spite of these changes, the extent of tissue loss was reduced after rapid warming. Evidence has been obtained to indicate that this beneficial effect of rapid warming is brought about by maintenance of blood flow through capillaries. The high skin temperatures of frostbitten parts treated by rapid warming are indicative of high rates of blood flow through the tissues. The accelerated rate of entrance of fluorescein from the blood stream into the interstitial fluid of frostbitten ears following rapid warming was observed in contrast to the slow entrance of the dye in frozen untreated ears. Since evidence has been presented which indicates a direct relationship between rate of penetration and available diffusion area (8), the above observation may be interpreted to indicate the preservation of a larger number of functional capillaries, and hence larger diffusion area, in the rapidly warmed ear. Delay of capillary stasis following rapid warming of frostbitten ears has been observed with the microscope in transilluminated ears, and persistence of blood flow has been verified with the aid of injected carbon.

In a recent publication of the Red Army on cold injury Ariev (13) states: "The fundamental goal of treatment in the pre-reactive period is elevation of tissue temperature, that is, warming of the cold-injured extremity, leaving aside the question of the advisability of rapid warming as under war conditions it does not have a great practical importance." Girgolav (12) also concurs in this view: "It has been definitely ascertained through a number of experimental investigations on different animals, that the more rapidly the chilled animal or any of its parts, such as the ear or extremity, is warmed, the more effective is recovery. From numerous experiments of this type, it has been clearly demonstrated that warming is the most satisfactory procedure."[2] If immediate rapid warming is as effective a measure in the prevention of gangrene following cold injury in man as it appears to be in experimental animals it would be highly desirable to develop methods for the institution of this treatment in the field. In spite of Ariev's statement (13) that conditions for warming are seldom available at evacuation stations at the front and that therefore the question of rapid or slow warming is devoid of practical interest, it seems possible that equipment for rapid warming could be developed if circumstances warrant its use.

Although immediate rapid warming of frostbitten feet and ears results in far less tissue loss

[2] Although this paper by Girgolav is titled "Modern Data on Frostbite" it is apparent that he is also concerned with hypothermia. For example he speaks of "frozen animals" treated by rapid warming and describes the minimum body temperatures tolerated. Part of this confusion has undoubtedly arisen as the result of translation. In view of the results obtained by Ariev (1) with rapid warming of rabbit ears which were frozen beyond doubt, it appears probable that Girgolav intends, in the quotation cited above, to include both frostbite and hypothermia. Treatment of hypothermia by means of rapid warming is considered by Sheinis (14) and by Alexander (15).

than in untreated extremities, the ear or foot which is retained is always thickened by the presence of dense fibrous connective tissue. Later, contraction of the scar is particularly evident in ears, which become shortened, narrower, and somewhat distorted in appearance (Figure 1). With the aim of reducing the extent of this fibrosis which may lead to loss of function, rapid warming should be supplemented by measures designed to control edema. The application of pressure dressings or of closed plaster or plastic casts to feet are satisfactory methods of accomplishing control of the edema following experimental frostbite. These methods of treatment will be described in detail in another paper (16).

SUMMARY

Controlled cold injury of rabbit feet and ears, produced by liquid immersion, was treated by immediate rapid warming of the injured part. The rapid warming consisted of immersion of the ear or foot in water at + 42° C. for 2 and 5 minutes respectively, immediately after withdrawal from the freezing mixture. In the majority of cases this treatment resulted in complete preservation of the frostbitten ear after immersion at − 55° C. for 1 minute, a severity of injury invariably leading to gangrene and loss of the injured part in untreated animals. Gangrene was in most cases prevented, or very greatly reduced in extent, and the amount of tissue loss was greatly reduced by rapid warming of rabbit feet injured by immersion at − 55° C. for 3 minutes or at − 15° C. for 60 minutes. Evidence is presented to indicate that the beneficial effect of rapid warming is brought about by alteration in the pattern of local blood flow in the frostbitten part.

Several weeks after injury the treated ears were shrunken and somewhat thickened and the treated feet showed evidence of periarticular fibrosis sufficient to cause spreading of the feet and toes. It is suggested that rapid warming should be supplemented by measures designed to control edema.

BIBLIOGRAPHY

1. Ariev, T. Y., Slow *vs.* rapid warming of frozen extremities. Vestn. Khirur., 1939, 57, 527.
2. Sonnenburg, E., and Tschmarke, P., Die Verbrennungen und die Erfrierungen. Neue Deutsche Chir., 1915, 17, 1.
3. Harkins, H. N., The Treatment of Burns. C. C. Thomas, Springfield and Baltimore, 1942.
4. Larrey, D. J., Surgical Memoirs of the Campaigns of Russia, Germany and France. Trans. by J. C. Mercer. Carey and Lea, Philadelphia, 1832.
5. Harkins, H. N., and Harmon, P. H., Thermal injuries: the effects of freezing. J. Clin. Invest., 1937, 16, 213.
6. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. I. A method for producing gangrene by means of controlled injury by cold. J. Clin. Invest., 1947, 26, 229.
7. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury. II. General course of events in rabbit feet and ears following untreated cold injury. J. Clin. Invest., 1947, 26, 236.
8. Crismon, J. M., and Fuhrman, F. A., Studies on gangrene following cold injury. V. The use of fluorescein as an indicator of local blood flow: fluorescein tests in experimental frostbite. J. Clin. Invest., 1947, 26, 268.
9. Crismon, J. M., and Fuhrman, F. A., Studies on gangrene following cold injury. VI. Capillary blood flow after cold injury, the effects of rapid warming, and sympathetic block. J. Clin. Invest., 1947, 26, 468.
10. Webster, D. R., Woolhouse, F. M., and Johnston, J. L., Immersion foot. J. Bone and Joint Surg., 1942, 24, 785.
11. Frey, S., Die örtlichen Erfrierungen im Kriege. Med. Klin., 1942, 38, 1067.
12. Girgolav, S. S., Modern data on frostbite. Am. Rev. Sov. Med., 1944, 1, 437. Trans. from Klin. med., 1943, 21, 3.
13. Ariev, T. Y., Basic features of the present day knowledge of frostbite. Medgiz, Moscow, 1943, pp. 43.
14. Sheinis, V. N., Freezing (generalized chilling). Medgiz, Moscow, 1943.
15. Alexander, L., The treatment of shock from prolonged exposure to cold, especially in water. Office of the Publication Board, Department of Commerce, Report No. 250, Washington (not dated).
16. Crismon, J. M., and Fuhrman, F. A., Studies on gangrene following cold injury. VIII. The use of casts and pressure dressings in the treatment of severe frostbite. J. Clin. Invest., 1947, 26, 486.

STUDIES ON GANGRENE FOLLOWING COLD INJURY. VIII. THE USE OF CASTS AND PRESSURE DRESSINGS IN THE TREATMENT OF SEVERE FROSTBITE[1]

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It has been reported in a previous communication (1) that increased leakage of fluid from capillaries is an invariable consequence of cold injury in rabbits' ears and feet. Although stasis in the true capillaries was observed to occur in ears about 10 minutes after injury, it could be delayed by several procedures (2). The delayed appearance of stasis in the treated ears, as well as the invariable late occurrence of stasis at the junction between the normal and frostbitten regions of an ear, suggest that distortion of the tissues, resulting from the massive edema, may close true capillaries and produce stasis. The effect of capillary closure in limiting the exchanges between the blood stream and the cells is an important one.

The similarity between the vascular lesion in frostbite and in burns is obvious and has been pointed out previously by others (3). Glenn (4) has recently reviewed the development of the treatment of burns by means of external pressure and by means of non-elastic, non-distensible dressings. Whether the good results of these forms of treatment depend upon "the control of capillary leakage" as suggested by Glenn or whether the benefit may derive from prevention of the mechanical distortion produced by swelling has not been determined. However, measures which aim at the control of either of these consequences of heat injury find equal justification for their use in the treatment of frostbite.

Two different types of dressings are available for the mechanical control of fluid loss following injury:

(*a*) Closed plaster casts were used by Glenn, Gilbert, and Drinker (5) for the treatment of experimental burns. The rationale of this procedure has been described by Glenn (4): "As plasma escapes from the dilated hyperpermeable capillaries, the extravascular tissue fluid pressure quickly builds up against the rigid encasement to exactly equal the pressure attempting to push fluid out of the capillaries." Such dressings should be skin-tight, non-elastic and non-distensible. In order to be maximally effective they should be applied before swelling of the injured part has taken place, and they must be applied in such a way that the distal part of the limb is encased in the dressing.

(*b*) External pressure to control fluid loss, as used by Allen and Koch (6) and others, is applied in the form of a pressure dressing of elastic material. A layer of padding separates the tissue from the elastic bandage and serves to equalize the pressures within the dressing. This form of dressing offers no obvious advantages over rigid casts for immediate treatment of frostbite in the period before swelling has begun.

METHODS

Standard cold injury. Injury was produced by immersing the hind feet of rabbits in a mixture of water, ethylene glycol, and alcohol cooled by the addition of solid carbon dioxide. The times of exposure and the temperatures used included those from 15 to 60 minutes at − 15° C. and 2 and 3 minutes at − 55° C. Details of standardization of cold injury produced by this means are given in the first paper of this series (7).

Closed plaster and plastic dressings. Closed plaster casts were prepared by impregnation of 1-inch gauze strips with dental "flasking" plaster. These were wetted and applied over a thin layer of gauze.

In order to prepare a light weight, non-distensible dressing which would serve a purpose similar to that of closed plaster casts, dressings were made from solutions of plastic materials applied to gauze. Most of the closed plastic dressings were made of a solution of polyvinyl butyral in isopropyl alcohol and castor oil ("Sealskin").[2] Some were formed with acetone solutions of 40 per cent isobutyl methacrylate or methacrylate interpolymer.[3] Those made of "Sealskin" proved to be most satisfactory;

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

[2] "Sealskin—viscous," Clay-Adams & Co., N. Y.

[3] Kindly supplied by E. I. duPont de Nemours, Ammonia Dept., Wilmington, Del.

examples are shown in Figure 1. The dried cast was very light in weight, non-distensible, and non-elastic, but to some extent permeable to air. Those made from the methacrylates were more rigid, less permeable to air, and tended to shrink slightly after application.

In most cases closed plaster and plastic dressings were applied to the feet of rabbits after thawing had taken place, but before swelling was noticeable. Upon measurement of foot volume immediately before application of the cast it was usually found to have increased about 1 ml. In a few animals the casts were applied before thawing was complete. The results did not differ from those first described. All feet had been freed of hair by close clipping before injury. The alcohol-ethylene glycol-water solution (7) in which the frostbite was produced was removed from the foot by gentle wiping. Tincture of merthiolate was usually used as a surface antiseptic, although this was sometimes omitted. Next a thin layer of K-Y jelly or of petrolatum was applied. Each toe was separated from the next by means of gauze, cotton, paper wadding, or lamb's wool. A thin layer of the padding was also uniformly distributed around the foot. Over this was applied 1 layer of seamless tubular gauze, then 2 to 3 layers of 1-inch gauze followed by 2 layers of seamless tubular gauze. The plastic solution was applied with a wooden tongue depressor in 2 coats; 1 over the gauze strips and 1 over the final layer of tubular gauze. The feet were then dried for 20 to 30 minutes in the air stream from a fan. The plastic dressing covered the entire foot and extended as far proximally as possible without impairing ankle flexion.

Pressure dressings. Pressure dressings were always applied after swelling was well started or after it had reached a maximum. They were constructed of approximately 2.5 yards (stretched length) of 1-inch wide elastic bandage. Both "Ace" and "Adaptic" bandages were used; standard 2-inch widths were cut lengthwise before use. Padding material was applied in a thicker layer than was used under closed plastic dressings. Of the 30 pressure dressings used, 24 were padded with lamb's wool, 4 with cotton and 1 each with paper wadding or gauze. Care was taken to begin tight application of the bandage distally and work as far toward the ankle as full flexion of this joint would permit.

RESULTS OBTAINED BY THE USE OF CLOSED PLASTER AND PLASTIC DRESSINGS AND THEIR COMBINATION WITH WARMING AND COOLING

For convenience of comparison of results following various types of treatment with appropriate untreated controls, the following classification of tissue loss in the order of increasing severity was used (Table I):

I. Partial loss of the toes.
II. Complete loss of the toes.
III. Loss of all tissue distal to a line 1.5 cm. from the level of frostbite.
IV. Loss of all tissue distal to a line 1.0 cm. from the level of frostbite.
V. Loss of all tissue distal to a line 0.5 cm. from the level of frostbite.
VI. Loss of all tissue except a narrow extension of the plantar pad.
VII. Loss of all tissue to the level of frostbite.

Table I indicates in the first column the type of treatment, the time of exposure, and temperature of the freezing mixture used to produce injury.

Closed plaster and plastic dressings. Closed plaster casts were applied to rabbits' feet following 3-minute exposure at $-52°$ C. to $-55°$ C.; closed plastic dressings were applied following 3-minute exposure at $-53°$ C. to $-56°$ C., 2-minute exposure at $-54°$ C., and 60-minute exposure at $-15°$ C. The results as shown in Table I indicate the number of times each degree of tissue loss was encountered in untreated controls and treated animals. The efficacy of this form of treatment may be seen from comparison with data on similar untreated injuries. It is apparent that the most striking results were obtained when the treatment was applied to frostbite of 3-minute duration at $-55°$ C. (a severe injury, which usually resulted in complete loss of the foot to the level of frostbite). Of 7 animals, frostbitten for 3 minutes at $-55°$ C. and treated by means of plaster or plastic casts padded only with gauze, 6 animals lost only toes or parts of toes. A typical result of this treatment is shown in Figure 1. It is apparent that too early removal of casts is disastrous; those animals frostbitten 60 minutes at $-15°$ C. with casts removed after 2 and 3 days (see examples marked * in Table I) lost the entire injured region.

Rapid warming followed by closed plastic dressings. The feet of 6 animals were exposed at $-53°$ C. to $-55°$ C. for 3 minutes, then rapidly warmed in water for 5 minutes at $+42°$ C. and encased immediately in closed plastic (Sealskin) dressings padded with a minimum amount of lamb's wool. While without treatment none of the 17 animals subjected to this degree of injury retained more than 1.5 cm. of the injured part of the foot, all 6 treated by rapid warming plus plastic dressings lost only the toes or parts of the toes (Table I).

Closed plastic dressings [illegible]. Six [illegible]

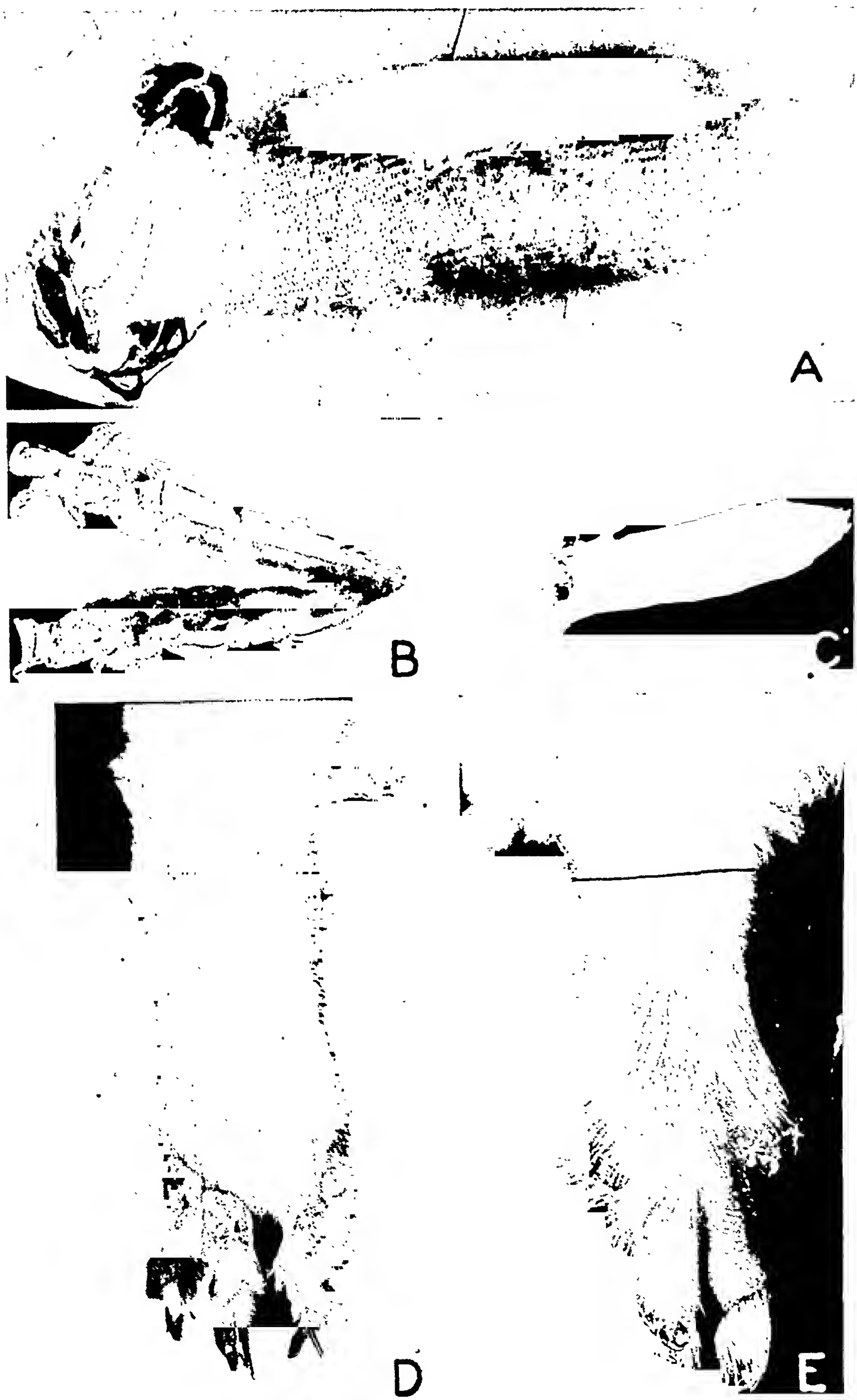

Fig. 1.

TABLE I

The loss of tissue sustained in rabbits' feet following various types of treatment of experimental frostbite

Treatment	Degree of tissue loss / Total number animals	Number of animals sustaining each degree of tissue loss						
		I	II	III	IV	V	VI	VII
Untreated controls								
3 min. at −55° C.	17	0	0	1	2	1	6	7
2 min. at −55° C.	5	0	4	0	0	0	1	0
60 min. at −15° C.	4	0	1	0	1	0	1	1
Closed plaster casts								
3 min. at −55° C.	4	1	3	0	0	0	0	0
Closed plastic dressings								
3 min. at −55° C.	9	3	0	0	0	0	4	2
2 min. at −55° C.	5	2	3	0	0	0	0	0
60 min. at −15° C.	4	1	1	0	0	0	0	2*
Rapid warming plus plastic dressings								
3 min. at −55° C.	6	1	4	0	0	0	1	0
Plastic dressings plus cooling								
3 min. at −55° C.	6	0	3	0	0	0	1	2
Rapid warming, plastic dressing, and cooling								
3 min. at −55° C.	5	1	1	0	2	0	0	1
Pressure dressing								
3 min. at −55° C.	7	0	4	0	0	0	0	3
2 min. at −55° C.	5	4	1	0	0	0	0	0
60 min. at −15° C.	3	3	0	0	0	0	0	0
Rapid warming plus pressure dressing								
3 min. at −55° C.	5	2	3	0	0	0	0	0
60 min. at −15° C.	3	3	0	0	0	0	0	0
Multiple incision plus pressure dressing								
3 min. at −55° C.	7	0	2	1	0	0	1	3
Pulsating pressure								
3 min. at −55° C.	13	0	1	0	2	1	0	9
2 min. at −55° C.	2	1	0	0	0	0	1	0

* The dressings were removed from the feet of these animals on the second and third day after injury.

II). Following 3-minute exposure of the foot at − 55° C., a closed plastic (Sealskin) dressing was applied, permitted to dry 15 to 20 minutes, and the foot was then cooled for 6 to 8 hours. Thermocouples were placed inside the dressings of rabbits 84, 88, and 98 in order that the temperature of the

FIG. 1. A. RABBIT FOOT ENCLOSED IN "SEALSKIN" PLASTIC CAST

Leads from thermocouples enclosed in cast are wound around the ankle.

B. "SEALSKIN" PLASTIC CAST REMOVED FROM RABBIT FOOT AND OPENED TO SHOW PADDING

C. METHACRYLATE PLASTIC CAST AFTER REMOVAL FROM RABBIT FOOT

D. RABBIT FOOT PHOTOGRAPHED 15 DAYS AFTER FROSTBITE FOR 3 MIN. AT −54° C.

Plastic cast applied after thawing and left in place for 7 days.

E. RABBIT FOOT PHOTOGRAPHED 18 DAYS AFTER FROSTBITE FOR 3 MIN. AT −54° C.

Pressure dressing applied 3½ hours after injury and left in place for 4 days. The level of immersion in the freezing mixture is indicated in D and E by the transverse inked line.

TABLE II

Treatment of frostbite by closed plastic dressings plus cooling

Feet exposed at −54° C. to −56° C. for 3 minutes. Closed plastic dressing applied after thawing. Cooling began immediately after dressing dried (about 15 minutes).

Animal number	Cooling	Days dressing left in place	Result
84*	Cooled to +5° to +15° C. for 6 hours	5	Lost to level of frostbite
87	Packed in ice 8 hours	5	Lost to level of frostbite
88*	Cooled to +1° to +5° C. for 7 hours	6	Lost toes
98*	Cooled to +10° C. 7 hours	5	Lost toes
103	Cooled in ice 7 hours	6	Lost to level of frostbite except 3 cm. tongue of tissue on plantar surface
104	Cooled in ice 7 hours	6	Lost toes

* Thermocouples placed inside dressing.

foot could be maintained at 5° to 10° C. during the cooling period. Three of these animals lost only the toes, while 3 lost an amount of tissue similar to that in untreated animals.

Rapid warming followed by closed plastic dressings and cooling. In 5 animals the treatments used above were combined. Immediately after injury (by exposure of the foot for 3 minutes at − 55° C.), the foot was warmed 5 minutes in water at + 42° C., encased in a plastic (Sealskin) dressing, and then cooled with ice during the next 5 to 6.5 hours (Tables I and III). This procedure did not seem to produce results superior to those obtained by closed dressings alone or by rapid warming followed by closed plastic dressings.

TABLE III

Treatment of frostbite by immediate rapid warming plus closed plastic dressings plus cooling

Feet exposed at −53° C. to −55° C. for 3 minutes, warmed in water at +42° C. for 5 minutes. Closed plastic dressing applied immediately after warming; cooling began immediately after dressing dried (about 15 minutes).

Cooling	Days dressing left in place	Result
Cooled at +5 to +12° C. for 5 hours	7	Lost distal and middle phalanges of all toes
Cooled in ice 6.5 hours	7	Lost to 1 cm. distal to level of frostbite
Cooled in ice 6 hours	7	Lost toes
Cooled in ice 6 hours	6	Lost to 1 cm. distal to level of frostbite
Cooled in ice 6 hours	6	Lost to level of frostbite

TEMPERATURE MEASUREMENTS OF FOOT ENCASED IN PLASTIC DRESSINGS

Method. In 11 animals thermocouples were placed on the toes or on the dorsum of the foot, or both, before application of the plastic dressings. The thermocouple leads were brought out through the proximal end of the dressing and were wrapped around the animal's leg, as shown in Figure 1, so as not to interfere with its movement in the cage when temperatures were not being measured. Temperature measurements were made with a Leeds and Northrup Potentiometer Indicator using iron-constantan thermocouples attached to copper discs 7 mm. in diameter.

In 6 of the animals the thermocouples were primarily for the purpose of measuring the temperature inside the dressings during cooling of the feet following injury. Measurements of the temperatures were, however, continued for the period during which the dressing was left in place.

In the remaining animals, temperature measurements were made during the first several hours after application of the dressing and at intervals during the ensuing 5 to 7 days. It was found that the temperature of the foot inside the dressing gave an accurate indication of the blood supply to the injured part. In those feet or parts of feet which were subsequently lost, the temperature (measured by the thermocouples within the dressing) either failed to rise above about 26° C. to 29° C. or fell to this level after 24 to 48 hours. Figure 2 shows temperature measurements of the toes and dorsal surface of the feet of 2 animals during the period that the dressings were in place. In both instances the feet were exposed for 3 minutes at − 55° C. Plastic (Sealskin) dressings were applied immediately after thawing occurred. In No. 32, padding was used between the toes and around the foot in such a manner that uneven pressure was avoided. The temperature of both the toes and dorsum increased rapidly and remained high throughout the period of measurement. Tissue loss was confined to 1 toe. In No. 30, no padding of any kind was used between the toes and it was afterward found that excessive pressure occurred around the base of the metatarsals. In this animal the temperature within the dressing increased more slowly, never exceeded 30° C., and fell again after 24 hours. Loss of the entire injured area with the exception of a narrow segment on the plantar surface occurred.

The incorporation of thermocouples for frequent measurement of skin temperature within closed dressings employed in human surgery might offer a convenient and accurate means of determining the state of blood flow to the part when it is not possible to determine this by direct observation.

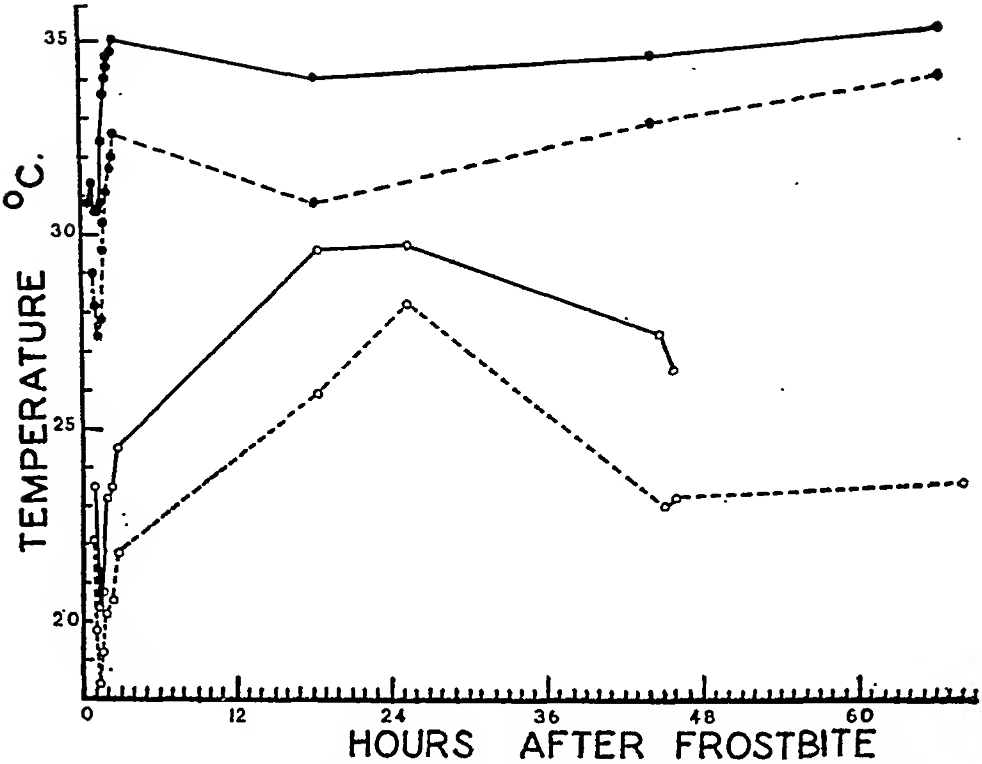

FIG. 2. TEMPERATURES OF RABBITS' FEET IN PADDED AND UNPADDED PLASTIC CASTS

Feet exposed for 3 minutes at − 55° C. Thermocouples placed on dorsum of foot and between toes before application of cast. Representative data from 2 animals.

Solid circles and solid line—temp. of dorsum in padded cast. Solid circles and broken line—temp. of toes in padded cast. Open circles and solid line—temp. of dorsum in unpadded cast. Open circles and broken line—temp. of toes in unpadded cast.

RESULTS OBTAINED BY THE USE OF ELASTIC PRESSURE DRESSINGS AND BY PRESSURE DRESSINGS IN COMBINATION WITH MULTIPLE INCISION OR RAPID WARMING

These dressings were, in every case, applied after swelling had reached its maximum. They offer the advantage, over closed plaster or plastic dressings, of not requiring immediate application.

Elastic pressure dressings. Elastic pressure dressings, without other treatment, were applied to the feet of rabbits injured by exposure for 3 minutes at − 55° C., for 2 minutes at − 54° C., and for 50 minutes at − 15° C. (Table I). The results should be compared with those obtained in untreated cold injury of similar duration. It is evident that the amount of tissue loss was reduced in most animals. Following 3-minute exposure at − 55° C., 4 out of 7 animals lost only the toes. With the 2 less severe degrees of cold injury, parts of the toes were saved in all except 1 animal.

Rapid warming followed by elastic pressure dressings. Rabbits' feet exposed for 3 minutes at − 53° C. to − 55° C. or for 60 minutes at − 15° C. were immediately warmed in water at + 42° C. for 5 minutes, then permitted to swell for 40 to 180 minutes before application of elastic pressure dressings. In all 8 animals the results were uniformly good (Table I). Following 3-minute exposure at − 55° C., parts of the toes were saved in 2 animals.

Multiple incision followed by elastic pressure dressings. The method of multiple incision, introduced by Noesske (8), was applied to frostbite by Wittek (9) and Bundschuh (10). Although their results may be questioned on the grounds of inadequate numbers of control cases, the removal of fluid and the establishment of free drainage before application of pressure dressings may seem to be desirable. Seven animals were treated in this manner following 3-minute exposure at − 55°

C. (Table I). In the first 6 animals, 3 incisions were made through the skin of the dorsal surface parallel to the long axis of the foot. In the seventh animal similar incisions were made on the toes. In order to encourage free drainage the incisions were packed with a small amount of 5 per cent sulfathiazole cream before application of the pressure dressings. Although the animal in which the toes were incised lost only the toes, the results are not superior to those obtained by means of pressure dressings alone. The added danger of infection probably constitutes sufficient grounds for avoiding this method of treatment in clinical practice.

TREATMENT OF COLD INJURY BY PULSATING EXTERNAL PRESSURE

Pressure pulses transmitted through tissues from arteries and arterioles have been shown to be one of the important dynamic agencies in the maintenance of lymph flow (11). To the extent that edema following severe injury by cold (1, 2) may be responsible for some of the changes leading to gangrene, the acceleration of lymph drainage of edematous areas should be helpful in reversing the processes which lead to the development of gangrene. Even in circumstances where gangrene does not occur, the swelling has been shown to lead to induration and later formation of collagenous scars in the subcutaneous tissues (12). Past experience has shown that most cases of accidental injury by cold, especially in military operations, do not receive treatment until a considerable time after exposure, and edema is then usually well advanced. The literature has been reviewed in the first paper of this series (7).

The experiments below were undertaken to study the effects of external application of pulsating pressure upon the course and ultimate results of severe injury by cold in the feet of rabbits.

Methods

Fifteen rabbits were used in the study. Thirteen of the animals were subjected to a degree of injury by cold which had been shown to produce extensive gangrene (Table I); 3 minutes at $-50°$ C. to $-56°$ C. The remaining 2 animals received injury to the feet which usually resulted in some gangrene and loss of the toes: 2 minutes at the same temperatures used above.

Treatment was begun at varying times after injury. In all cases swelling was allowed to progress practically to its maximum. The times of application of treatment are indicated in Table IV.

External pulsating pressures were applied to the injured feet by enclosing them in a heavy glass cylinder having an internal diameter of 2 inches. The cylinder was filled with water and connected to the source of pressure with heavy-walled pressure tubing. Various methods were used to maintain a closed system with the foot inside the cylinder. The first method involved the use of a heavy rubber diaphragm, having a hole 2 cm. in diameter in its center. A short cuff of thin-walled latex drain tubing was cemented to the edges of the hole and extended at right angles to the plane of the diaphragm. The hole through the diaphragm and the lumen of the cuff were distended by stretching, and the foot was introduced in such a way that the diaphragm enclosed the leg just above the ankle joint and the cuff extended distally along the foot. The free margins of the diaphragm were then secured in the manner of a gasket to the end of the glass cylinder and held in place by a ring plate and bolts. Because of some fear that the closure about the rabbit's legs might occlude venous blood flow or lymph drainage, the tight latex rubber cuff was replaced by a looser one extending proximally from the diaphragm: this was secured about the part of the leg proximal to the diaphragm by a collar of sponge rubber. The first 4 animals listed in Table IV were secured in the apparatus for pulsation by the first method of closure described. The next 3 animals were secured by means of the closure with a sponge rubber collar. The remaining 5 animals were given pulsation treatment under conditions which effectively prevented occlusion of blood flow or lymph drainage by the use of a dipped latex bag with a reinforced collar at the open end. The bag was introduced into the glass cylinder and the open end everted over the open end of the cylinder. This formed a closed system having a flexible portion invaginated to receive the foot.

Two types of apparatus were used as sources of pulsating pressure. Apparatus "A" (Table IV) consisted of a perfusion pump, artificial peripheral resistance, and air chamber. Propulsion of fluid by the pump was effected by the compression and forward movement of fluid in a segment of rubber tubing by 4 brass rollers mounted on the periphery of a rotating drum. Mean pressure level was determined by the compression pressure in a standard artificial peripheral resistance of the Starling type. The frequency of pulsations was determined by the rate of rotation of the drum and the size of the pressure pulses by the degree of compression of the rubber tubing by the rollers.

Apparatus "B" consisted of a pump constructed from a 5-ml. syringe mounted on a universal joint. The plunger of the syringe was moved by the arm of a crank driven through reduction gears. An adjustable air chamber constructed from a 50-ml. syringe provided for variable damping of the pulse waves. Frequency of pulsations was altered by changing the drive to the reduction gears, and the volume of the pulse was adjusted by manipulation of the stroke of the syringe plunger.

TABLE IV

Treatment of frostbite by means of pulsating external pressure

Animal no.	Time elapsed before treat.	Pressure	Rate	Duration of pulsation	Result
	hours	*mm. Hg*	*puls. per min.*	*hours*	
Feet exposed at −50° C. to −55° C. for 3 minutes					
9	24	+110/+90	208	7	Lost to level of frostbite
11	3	+111/+91	208	3	Lost to 1 cm. distal to level of frostbite
13	2	+90/+64	208	2.5	
	22	+90/+64	208	8	Lost to level of frostbite
35	3	+41/+34	208	3	Lost to 1/2 cm. distal to level of frostbite
54	4	+42/+38	208	2.5	Lost toes
63	3	+45/+35	208	2.5	Lost to level of frostbite
87	2	+30/−10	60	3	Lost to level of frostbite
103	1.5	+50/+10	60	5.5	Lost to level of frostbite
116	1	+40/−30	60	5	
	22	+40/−30	60	5	Lost to level of frostbite
86	1	+69/+12	60	5.5	Lost to level of frostbite
106	2	+27/−43	60	5.5	Lost to 1 cm. distal to level of frostbite
133	3.6	+10/−10	24	3.5	
	24	+10/−10	24	4	Lost to level of frostbite
138*	2	+12/−8	24	5	Lost to level of frostbite
Feet exposed at −55° C. to −56° C. for 2 minutes					
133	1.5	+20/−22	24	4.5	Lost to level of frostbite except 3 cm. tongue of tissue on plantar surface
119	1.5	+30/−30	24	5	Lost toes except first phalanx of 2nd. and 3rd.

* Frostbite followed by rapid thawing in water at +42° C. for 5 minutes.
Nos. 9, 11, 13, 35—Apparatus "A," tight cuff of latex drain tubing.
Nos. 54 and 63—Apparatus "A," sponge cuff.
No. 87—Apparatus "B," sponge cuff.
Nos. 103, 116, 86, 106, 133, 119, 138—Apparatus "B," no cuff.

The type of apparatus used for the treatment of the various animals is indicated in Table IV.

Results

The pertinent data from these experiments are presented in Table IV. The pulsation rates and the magnitudes of the pulse waves in the first 7 animals were selected to reproduce rather closely the pulsations to be expected in the small arteries of the extremities. The frequencies and magnitudes of pulse waves in the last 6 animals were employed to avoid possible interference with blood flow which might have been encountered with the use of higher pressures and also in order to reproduce influences upon lymph flow which resembled those resulting from muscular activity.

None of the feet treated by external pulsating pressure was found to decrease in volume during the period of treatment. In the interval from 15 to 21 hours after the foot was removed from the pressure cylinder, there was usually some additional swelling amounting to 1 to 3 ml. The time course of decreasing foot volume was usually a little more rapid than that of the untreated controls, but the difference was neither marked nor uniform.

The final result of the injury did not differ from that in untreated animals. Exposures of 3-minute duration to the freezing mixture at − 50° C. to − 56° C. produced gangrene and loss of the foot to within a few millimeters of the line of immersion, and 2-minute exposures to these temperatures was followed by loss of the toes. The time interval between the exposure to cold and the loss of tissue was the same as that in the controls.

STATISTICAL SUMMARY OF RESULTS

Analysis of the results was carried out by the "exact" method of Fisher (13) for 4-fold tables. The criterion of comparison of control animals with treated animals depended upon the distribution of degrees of tissue loss in the controls. None

of the control animals fell into class I or II (partial loss of toes or complete loss of toes). Therefore, the treated animals were compared with the controls on the basis of the number of animals in each series which fell into tissue loss classification I and II as distinguished from those falling into classes III to VII. Thus, for purposes of statistical analysis, no result was regarded as favorable unless the loss of tissue was restricted to the toes or parts of toes. Only the data from animals injured by exposure for 3 minutes at − 50° C. to − 56° C. were included in the statistical analysis. The values of P in Table V indicate for each type of treatment the probability of encountering the indicated reduction in tissue loss by chance. The various treatments are arranged in the order of their effectiveness in preventing gangrene. The probability values indicate that significant degrees of benefit were achieved by the use of non-distensible dressings and by pressure dressings, but favorable results were even more frequently encountered when these dressings were combined with other treatments, especially immediate rapid warming.

TABLE V

Statistical summary of results[4]

Treatment	Number of animals	P
Rapid warming and plastic cast	6	0.000020
Rapid warming and pressure dressing	5	0.000038
Plaster cast	4	0.000170
Pressure dressing	7	0.003300
Plastic cast and cooling	6	0.011000
Plastic cast	9	0.032000
Rapid warming, plastic cast, and cooling	5	0.034000
Multiple incision and pressure dressing	7	0.076000*
Pulsating pressure	13	0.433300**

* Difference from controls of doubtful statistical significance.
** Difference from controls not statistically significant.

DISCUSSION

The methods of treatment of cold injury described in this report have in common the attempt to control fluid loss from the blood stream in the region injured by cold. As measures of prevention, control has been effected through the application of non-distensible dressings before swelling occurred; as aids to the removal of edema fluid, elastic pressure dressings, the incision of swollen tissues, and the application of external pulsating pressure have been employed. The results show that prevention of swelling or the reduction of it by means of elastic pressure dressings is capable of altering the course of changes in tissues severely injured by cold. In contrast to the complete loss from gangrene of the part subjected to injury when no treatment is used, there is a striking reduction in the extent of gangrene following the application of a non-distensible dressing before swelling has occurred or the use of an elastic pressure dressing after swelling is maximal. In general, the amount of tissue saved by the use of measures designed to control swelling are somewhat smaller than the proportion saved by immediate, rapid warming (12). However, the best results obtained with pressure dressing or with casts are equal to those following rapid warming in terms of amount of tissue saved and much superior to those obtained by rapid warming in terms of functional recovery. Cold-injured tissues which escape gangrene and survive following treatment by immediate, rapid warming are thickened by massive induration in the early healing period and later by dense accumulations of collagenous scar tissue. If the injuries are treated by casts or pressure dressings, alone or in addition to rapid warming, induration and accumulations of scar tissue are insignificant.

The success or failure of treatment of cold injury by the application of casts or pressure dressings depended to an important degree upon the manner of application of the dressing and also upon the nature of the material used for padding. Unequal distribution of pressure, especially over bony prominences, contributed greatly to the development of gangrene. The irregular contours of the rabbits' feet, after removal of the hair by clipping, proved to be extremely difficult to pad evenly. Of the various materials tried as padding (paper wadding, absorbent cotton, gauze, and lamb's wool), the wool was the most satisfactory in both casts and pressure dressings. Both paper and cotton tended to become matted and stiff with absorbed exudate, while the lamb's wool without losing resilience permitted the continued drainage of exudate. The almost invariable loss of the toes following the severest injury (exposure of 3 *minutes* at − 55° C.) and the partial loss of

[4] See text for basis of comparison and method of computing values for P.

toes following the less severe degrees of injury may be attributed in part to unsatisfactory application and maintenance of pressure in these parts of the feet. The relationship between the mass and the surface area of the toes tended to facilitate their reaching a lower temperature during exposure, and the larger number of arteriovenous anastomoses contributed to early stasis by diverting blood flow away from the true capillaries (2). The above factors and undoubtedly others, as yet undefined, combine to determine the greater susceptibility of the toes to cold injury.

The results of treatment of cold injury by early mechanical control of the edema, by immediate rapid warming (12), and by the use of heparin as described by Lange and Boyd (14) all confirm the opinion of Bigelow (15) that stasis is not necessarily an irreversible process. The interruption of the transition from temporary arrest of blood flow by stasis to permanent arrest by thrombosis may be accomplished by a variety of means. In spite of the apparently diverse mechanisms of action of these methods of treatment, they have in common the essential feature that all of them must be applied early and continuously over an appreciable time period in order to be effective.

It should be pointed out that the "reversal of stasis," in the literal sense of restoration of blood flow through the identical capillaries once occluded by packed red blood cells, is not a necessary condition determining the survival of tissues severely injured by cold. However it may be accomplished, the necessary condition is the supply of oxygen and nutrients and the removal of metabolites. Resting tissues are maintained under normal conditions with blood flowing through only a very few of the total number of capillaries available, the flux of tissue fluid being adequate for the supply of cells remote from open capillaries (16). If such a flow of fluid can be maintained for a sufficient time in tissues severely injured by cold, new capillary channels may grow to replace those obliterated, initially by stasis and later by thrombosis.

In untreated experimental frostbite, the development of massive edema is accompanied by rising interstitial fluid pressure (17). When the edema reaches its maximum (about 2 hours after the severest forms of injury described in this report) the interstitial fluid pressure is 25 cm. of water. Not only is the rate of filtration sharply limited by the high counterpressure, but many of the minute vessels become closed, both by the rise of pressure surrounding the vessel above that within the lumen and by kinking as the tissues are distorted by edema. An additional harmful consequence of edema is the increased distance over which diffusion exchanges must be accomplished between remaining capillaries and surviving cells.

It is probably safe to assume that the fluid dynamics are essentially the same inside the non-distensible dressings used for the treatment of frostbite as those described by Glenn, Gilbert, and Drinker (5) when such dressings were used for the treatment of experimental burns. Even though a high rate of fluid loss from capillaries may persist, the rigid dressing enforces a uniform rise of pressure in all parts of the foot enclosed within the cast; spatial distortion is prevented and rapid removal of fluid is effected via the lymphatics as well as by seepage through the interstitial compartment. The mechanism of action of elastic pressure dressings is conceived to be fundamentally similar with the exception that such dressings are applied after swelling and hence have, in the early phases, a dynamic rather than a passive role in removal of fluid.

In untreated experimental frostbite, the skin temperature of injured feet of rabbits declined gradually over a period of about 48 hours until the temperature was approximately that of the environment (1). When skin temperature measurements were made on casted feet by means of thermocouples on the skin and enclosed in the cast, it was found that those parts which survived the injury remained warm while those destined to become gangrenous in spite of the treatment suffered the usual fall in temperature. It therefore seems evident that arrest of the usual process of progressive vascular occlusion is in some way brought about by the cast or pressure dressing. Removal of dressings as early as the third or fourth day after injury resulted in gangrene and loss of the entire injured region. Thus, the interruption of the injury process does not become stabilized until the dressings have been left in place for 5 to 7 days. While the exact nature of the change is not known, the time relations are at least consistent with the following hypothetical course of events: (a) primary stasis of true capillaries following the

initial injury, (*b*) persistence of filtration from arteriolar-venular capillaries (16) in amounts sufficient to maintain cells which survive the original injury, (*c*) the growth of new capillaries in sufficient numbers to provide for fluid removal and maintenance of cells by the time the dressings are removed.

SUMMARY

Prevention of gangrene following experimental frostbite in the hind feet of rabbits was attempted by mechanical control of edema with casts and pressure dressings. Combinations of rapid warming and subsequent cooling with casts and pressure dressings were also studied.

The injuries were of sufficient severity (immersion in liquid for 3 minutes at − 55° C.) to produce loss of virtually all of the exposed part of the foot in control animals. In similarly injured animals, treated by the above methods, marked improvement was noted. Gangrene was restricted to the toes or parts of the toes in a highly significant number of cases.

The most favorable results were obtained by the use of rapid warming followed either by casts applied before swelling occurred or by pressure dressings applied after maximum swelling. Casts or pressure dressings alone produced significantly favorable results. The maintenance of low skin temperatures (5° to 10° C.) in casted frostbitten feet produced better results than did casts alone, but the difference was slight. Multiple incisions through the skin of the injured regions, followed by the application of pressure dressings, led to survival of more tissue in the frostbitten feet than was observed in the controls; but the low statistical significance of the difference suggests that the procedure is of doubtful value. External application of pulsating pressure was completely ineffective in preventing gangrene.

Various factors influencing the successful use of the treatments have been discussed and a possible mechanism by which control of edema may prevent gangrene was suggested.

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BIBLIOGRAPHY

1. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury: II. General course of events in rabbit feet and ears following untreated cold injury. J. Clin. Invest., 1947, 26, 236.
2. Crismon, J. M., and Fuhrman, F. A., Studies on gangrene folowing cold injury: VI Capillary blood flow after cold injury, the effects of rapid warming and sympathetic block. J. Clin. Invest., 1947, 26, 468.
3. Harkins, H. N., The Treatment of Burns. C. C. Thomas, Springfield, Ill., 1942.
4. Glenn, Wm. W. L., A physiological analysis of the nature and of the treatment of burns. Ann. Surg., 1944, 119, 801.
5. Glenn, Wm. W. L., Gilbert, Helen H., and Drinker, C. K., The treatment of burns by the closed plaster method, with certain physiological considerations implicit in the success of this technique. J. Clin. Invest., 1943, 22, 609.
6. Allen, H. S., and Koch, S. L., The treatment of patients with severe burns. Surg., Gynec. and Obstet., 1942, 74, 914.
7. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury: I. A method for producing gangrene by means of controlled injury by cold. J. Clin. Invest., 1947, 26, 229.
8. Noesske, H., Zur Prophylaxe und Therapie drohender Fingergangrän bei Raynaudsche Krankheit. Münch. med. Wschr., 1909, 56, 2419.
9. Wittek, A., Zur Behandlungen der Erfrierungen. Münch. med. Wschr., 1915, 62, 416.
10. Bundschuh, E., Ueber Behandlungen der Erfrierungen. Münch. med. Wschr., 1915, 62, 416.
11. Gesell, R., The relation of pulsation to filtration. Am. J. Physiol., 1914, 34, 186.
12. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene following cold injury: VII. Treatment of cold injury by means of immediate rapid warming. J. Clin. Invest., 1947, 26, 476.
13. Fisher, R. A., Statistical Methods for Research Workers, par. 21.02. Oliver and Boyd, Edinburgh, 1936.
14. Lange, Kurt, and Boyd, L. J., The functional pathology of frostbite and the prevention of subsequent gangrene. Surg., Gynec. and Obstet., 1945, 80, 346.
15. Bigelow, W. G., Modern conception and treatment of frostbite: survey and discussion of literature. Canad. M. A. J., 1942, 47, 529.
16. Zweifach, B. W., The structural basis of permeability and other functions of blood capillaries. Cold Spring Harbor Symp. Quant. Biol., 1940, 8, 216.
17. Fuhrman, F. A., and Crismon, J. M., Studies on gangrene folowing cold injury: III. Edema following cold injury: its magnitude and the composition and source of edema fluid. J. Clin. Invest., 1947, 26, 245.

STUDIES OF THERMAL INJURY. VI.[1] HYPERPOTASSEMIA CAUSED BY CUTANEOUS EXPOSURE TO EXCESSIVE HEAT

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INTRODUCTION

That the rapid release of potassium from erythrocytes during an episode of generalized cutaneous hyperthermia may cause an increase in plasma potassium sufficient to contribute to the occurrence of circulatory failure and death was suggested by Schjerning in 1884 (1). The suggestion was based in part on the fact that postmortem examination of extensively burned persons often disclosed evidence of severe intravascular hemolysis and in part on the fact that a sufficient amount of potassium may be released from erythrocytes *in vitro* to raise the plasma concentration of that element to a level incompatible with continued cardiac function.

In a foregoing study (2) in this series, it was observed that generalized cutaneous exposure to excessive heat may lead to rapidly fatal circulatory failure. In several pigs that died in this manner and whose deaths were preceded by electrocardiographic evidence of severe disturbances in cardiac function, the plasma potassium was found to be increased to levels ordinarily considered incompatible with life. The implication of this observation was such as to warrant further study of the effects of hyperthermia on the potassium concentration of the plasma.

EXPERIMENTAL PROCEDURE

Samples of blood for chemical analysis were obtained from the heart by means of an inlying jugular cannula. Potassium determinations were carried out on the trichloroacetic acid filtrate of plasma and lysed blood according to the method of Lowry and Hastings (3) as modified by Cohn and Tibbetts. Hematocrit was determined in Wintrobe tubes after centrifuging for 30 minutes at 2,500 r.p.m. The method of Bing, *et al* (4), as modified by Ham (5) was used for determining plasma hemoglobin. Whole blood hemoglobin was determined on 0.1 ml. of 1/5 dilution of blood in 5 ml. dilute ammonia by the Klett-Summerson colorimeter.

Before undertaking further investigation of the relationship of hyperthermia to the development of hyperpotassemia, an experiment was undertaken to determine the effect of systemic anoxia on the potassium concentration of the plasma independently of hyperthermia.

A control sample of blood was taken from an 8.2 kgm. pig. The trachea was then exposed and clamped and 4 and 8 minutes later, additional samples of blood were obtained. The animal died at the end of 8 minutes and was allowed to remain on the operating table at room temperature for an hour thereafter, at which time, the fourth and last sample of blood was withdrawn. The analytical results are shown in Table I.

TABLE I

Changes in the blood of a pig during and after death by strangulation

Blood withdrawn	Volume packed cells	Hemoglobin in cells	Hemoglobin in plasma: Hemolysis	Potassium in red cells	Potassium in plasma
		grams per 100 ml.	*per cent*	*meq. per L.*	*meq. per L.*
Control	45	33	0	132	5.2
0 min.	Trachea clamped				
4 min.	49	31	0	128	9.1
8 min.	48	34	0	130	9.3
8 min.	Animal died				
68 min.	?	?	0	?	16.8

It may be seen that the plasma potassium level was almost doubled during the 8 minutes that elapsed between the onset of asphyxia and death. Most of the increase occurred during the first 4 minutes of this period. There are two obvious sources from which the increment may have been derived, one being the erythrocytes and the other the extravascular tissue. A comparison of hematocrit and hemoglobin content of cells at the end of the 4-minute period indicates that swelling of erythrocytes had occurred. The hematocrit rose from 45 to 49, whereas the hemoglobin dropped from 33 to 31 grams per 100 ml. of cells. It appears that the observed decrease in the con-

[1] This work has been done under Contract NDCrc-169 between the President and Fellows of Harvard College and the Office of Scientific Research and Development who assume no responsibility for the accuracy of the statements contained herein.

centration of intracellular potassium from 132 to 128 meq. per L. was probably due to this swelling of red cells rather than to loss by leakage. Since the actual potassium content of the erythrocytes did not appear to have dropped and since there was no hemolysis, it was inferred that the potassium in the plasma had been increased by diffusion from extravascular sources.

The need for taking blood promptly after death, if reliance is to be placed on analytical results, is illustrated by the rise in plasma potassium that occurred during the first hour postmortem. At death, the plasma concentration of potassium was 9.3 meq. per L., whereas 1 hour later it was 16.8. Although there is no evidence in the data presented in Table I as to the source of the postmortem increment in plasma potassium, unpublished studies indicate that both leakage from red blood cells and diffusion from extravascular tissues may cause a postmortem rise in plasma potassium. So far as the significance of this experiment in providing control data is concerned, it is apparent that a two-fold rise in plasma potassium may occur as a result of severe systemic anoxia independently of hyperthermia.

In order to correlate chemical data with known degrees of cutaneous hyperthermia, it was decided to sub-

TABLE II

Pig no.	Time	Thermal exposure	Body temperature	Time of death	Blood samples time taken	Volume packed cells	Hemoglobin in cells	Hemoglobin in plasma: Hemolysis	Potassium in red cells	Potassium in plasma: Total	Change	Potential increment from hemolysis	Increment from sources other than hemolysis
	minutes		*°C.*				*grams per 100 ml.*	*per cent*	*meq. per L.*	*meq. per L.*			
877	Control		34.3		Control	32	29	0.0	145	3.8			
	0	Started											
	10				10M	33	30	0.0	158	6.2	+2.4	0	2.4
	14	47°			14M	33	30	0.0	154	6.9	+3.1	0	3.1
	24		44.3		24M	31	32	0.0	158	8.2	+4.4	0	4.4
	26	Stopped		+									
1057	Control		37.0		Control	35	35	0.1	115	4.4			
	0	Started											
	20	47°			20M	36	35	0.0	125	7.0	+2.6	0	2.6
	36	Stopped	45.5	+	36M	36	35	0.2	120	10.2	+5.8	0.2	5.6
1056	Control		37.8		Control	33	37	0.1	118	4.7			
	0	Started											
	10				10M	33	36	0.1	114	5.9	+1.2	0	1.2
	15	47°			15M	35	35	0.1	113	7.2	+2.5	0	2.5
	34				34M	36	35	0.2	118	7.1	+2.4	0.1	2.3
	45	Stopped	45.5	+									
923	Control		?		Control	48	33	0.0	?	3.8			
	0	Started											
	13				13M	47	44	0.1	124	5.5	+1.7	0.1	1.6
	23				23M	46	46	0.2	120	5.5	+1.7	0.2	1.5
	34	47°			34M	55	32	0.1	113	6.2	+2.4	0.2	2.2
	42				42M	55	32	0.1	112	6.5	+0.3	0.2	2.8
	47				47M	56	33	0.1	?	7.5	+3.7	0.2	3.5
	50	Stopped	?	+									
899	Control		37.4		Control	38	34	0.4	139	3.6			
	0	Started 75°											
	1	Stopped											
	5				5M	48	31	3.6	109	10.2	+6.6	3.7	2.9
	16				16M	37	33	8.6	117	6.9	+3.3	6.5	
	46				46M	39	32	7.5	118	4.2	+0.6	6.2	
	76		39.2		76M	37	35	6.7	122	7.4	+3.8	5.2	
918	Control		36.6		Control	34	44	0.0	131	3.7			
	0	Started 75°											
	3	Stopped											
	4				4M	51	30	2.5	98	11.0	+7.3	2.6	4.7
	11				11M	45	42	4.4	110	9.5	+5.8	4.2	1.6
	17				17M	44	35	5.9	102	9.5	+5.8	5.1	0.7
	37		40.6		37M	40	48	5.6	103	9.4	+5.7	4.0	1.7
	55			+									

TABLE II—*Continued*

Pig no.	Time	Thermal exposure	Body temperature	Time of death	Blood samples time taken	Volume packed cells	Hemoglobin in cells	Hemoglobin in plasma: Hemolysis	Potassium in red cells	Potassium in plasma: Total	Potassium in plasma: Change	Potassium in plasma: Potential increment from hemolysis	Potassium in plasma: Increment from sources other than hemolysis
	minutes		*° C.*				*grams per 100 ml.*	*per cent*	*meq. per L.*	*meq. per L.*			
919	Control				Control	45	33	0.8	118	4.2			
	0	Started	37.1										
	4	75°			4M	56	29	7.8	81	25.5	+21.3	8.6	12.7
	5	Stopped											
	8				8M	47	26	25.5	67	21.4	+17.2	20.2	
	10				10M	40	32	22.2	?	18.3	+14.1	?	
	14				14M	35	37	23.1	77	17.0	+12.8	12.6	
	17		44.3		17M	33	31	30.1	72	17.5	+13.3	15.2	
	18			+									
913	Control		38.6		Control	26	37	0.0	116	3.5			
	0	Started											
	2	75°			2M	35	32	12.3	103	14.2	+10.7	7.7	3.0
	6				6M	32	33	24.5	96	17.7	+14.2	14.7	
	7	Stopped											
	8		40.8	+	8M	30	32	25.3	111	17.4	+13.9	16.0	
907	Control		37.3*		Control	42	34	0.6	125	3.5			
	0	Started											
	8	75°			8M	53	31	2.7	100	17.4	+13.9	3.1	10.8
	10	Stopped	42.5*	+									
910	Control		36.8		Control		?	?		3.0			
	0	Started											
	2				2M		?	?		19.1	+16.1		
	5	75°			5M		?	?		18.1	+15.1		
	7				7M		?	?		24.0	+21.0		
	13			+									
	14	Stopped	43.7		14M		?	?		17.3	+14.3		
908	Control		?		Control	32	?	?	106	3.8			
	0	Started											
	4				4M	?	?	?	?	16.7	+12.9		
	9	75°			9M	33	?	?	98	18.5	+14.7		
	11				11M	32	?	?	90	17.1	+13.3		
	14	Stopped	?	+									
912	Control		36.0		Control	33	37	0.0	125	4.1			
	0	Started											
	1				1M	45	31	1.9	102	16.7	+12.6	1.6	11.0
	4	75°			4M	33	37	19.2	?	?	?	?	
	5				5M	34	34	24.2	100	16.4	+12.3	16.5	
	10				10M	40	31	19.9	85	16.4	+12.3	14.2	
	14	Stopped	43.1	+									

* Right heart temperature.

merge animals in hot water rather than expose them to hot air. By the former method, the temperature of the surface of the skin could be controlled with greater precision than was possible by the latter.

The experimental procedure that was followed in submerging animals in hot water is described in detail in Study VII of this series (6). The animals were anesthetized with pentobarbital sodium and between 60 and 75 per cent of the total body surface was immersed in hot water. The effects on the blood of exposing 4 pigs in this manner at 47° C. and 8 pigs at 75° C. are shown in Table II.

RESULTS

Exposure at 47° C.: Although all of the [illegible] animals developed an acute and rapidly fatal systemic hyperthermia, none showed a rise in plasma potassium significantly greater than that which may result from anoxia independently of hyperthermia

In none of these was the magnitude of the increase comparable to that which was observed in some of the hot air exposures reported in Study IV of this series (2).

In the first two animals, it appeared that the potassium increase in the plasma was derived from extravascular sources. In one sample from the third animal the increase could have been due to leakage from red blood cells. In the fourth animal it could have been due in part to leakage from intact erythrocytes, and in part to diffusion from extravascular tissue. Cutaneous hyperthermia of the kind produced in these animals did not result in a significant amount of intravascular hemolysis.

Exposure at 75° C.: The chemical changes in this group were of a different order of magnitude than those observed in animals exposed at 47° C. All animals exposed for more than 3 minutes at 75° C. developed plasma potassium levels in excess of 16 meq. per L. In most instances, such levels were reached during the first few minutes of exposure and were either maintained or increased as the period of exposure was prolonged. If the pig survived for more than a few minutes after the termination of the exposure, there was a slow decline in plasma potassium concentration. Thus, in animal No. 919 the plasma potassium rose from 4.2 to 25.5 meq. during the first 4 minutes of exposure, and during the next 4 minutes declined to 17.4.

The rapidity with which an excessively high plasma potassium level may fall is indicated by the discrepancies that were observed between estimated increments by hemolysis and total amounts actually present in the plasma. Thus, it may be seen in the case of pig No. 913 that with an increment of 7 meq. per L. between the 2- and 6-minute samples by hemolysis, the actual plasma level rose by only 3.5 meq. Similarly, in pig No. 912 the increment by hemolysis between the 1- and 5-minute samples was 14.9 meq. per L. whereas the total plasma potassium actually changed from 16.7 to 16.4 during this period.

In most of the animals exposed at 75° C., there was some increase in the volume of packed cells. The comparison of cell volume and hemoglobin content indicated that most, if not all, of the early increase in cell volume was due to swelling of erythrocytes rather than to loss of plasma or mobilization of cells from storage depots.

It is of interest to note that plasma hemoglobin values as high as 24 per cent hemolysis were observed as early as 5 minutes after the onset of cutaneous hyperthermia. It was estimated that during this period the temperature in the vicinity of the most superficial blood vessels probably rose to approximately 70° C.

Chemical changes in the blood of dogs caused by cutaneous hyperthermia: It was inferred from the foregoing experiments on pigs that most of the potassium responsible for these potentially fatal plasma levels either leaked out of intact red blood cells or escaped from hemolyzed cells. If this inference is correct, fatal hyperpotassemia due to cutaneous hyperthermia would occur only in animals having a high concentration of potassium in the erythrocytes such as man or pig. Its occurrence could not be expected in an animal having a low cellular concentration of potassium as is the case in dog's blood.

To test this assumption, samples of blood were taken from each of 5 dogs before and during immersion in hot water. The results of these experiments are shown in Table III.

The animals were exposed at temperatures ranging between 55° and 75° C. until death occurred. The highest potassium concentration observed in the erythrocytes in control samples of blood from these animals was 9.4 meq. per L., in contrast to the pig whose erythrocyte concentrations ranged between 106 and 145 meq. per L. The greatest potassium increase that occurred in the plasma of the dogs that died as a result of cutaneous exposure to heat was from 3.9 to 8.2 meq. per L.

The increments to the plasma potassium that were observed in these animals could not be accounted for by loss of potassium from the erythrocytes. The potassium content of the red blood cells of the dogs characteristically rose during exposure in contrast to the loss of potassium that occurred from the erythrocytes of the pig. As in the case of the pig, there was severe intravascular hemolysis in animals exposed at 75° C. until death occurred.

It can be inferred, therefore, that the development of a potentially fatal level of hyperpotassemia following cutaneous exposure to heat results from the rapid release of potassium from thermally in-

TABLE III

Changes in blood of dogs caused by immersion in hot water

Dog no.	Time	Thermal exposure	Body temp.	Time of death	Blood samples time taken	Volume packed cells	Hemoglobin in cells	Hemoglobin in plasma: Hemolysis	Potassium in red cells	Potassium in plasma
	minutes	° *C.*	° *C.*				*grams per 100 ml.*	*per cent*	*meq. per L.*	*meq. per L.*
931	Control		35.4		Control	35	37	0	9.9	2.8
	0	Started								
	5				5M	41	36	0	8.1	5.2
	13	55°			13M	57	32	0	10.7	4.7
	21		41.4		21M	57	33	0	11.2	6.9
	23	Stopped		+						
930	Control		36.9		Control	49	34	0.1	4.3	4.0
	0	Started								
	5				5M	66	27	17.9	6.4	3.3
	8	60°			8M	65	28	20.2	5.5	4.7
	11		39.1		11M	62	28	23.8	6.1	5.3
	17	Stopped		+						
929	Control		37.2		Control	49	34	0.3	6.3	3.9
	0	Started								
	3				3M	57	29	26.1	7.0	4.8
	9	75°			9M	42	37	31.8	5.7	6.1
	13		44.1		13M	39	34	35.8	7.9	8.2
	14	Stopped		+						
922	Control		37.9		Control	42	35	0.2	8.8	3.1
	0	Started								
	3				3M	47	30	22.9	8.9	5.8
	7	75°			7M	47	30	29.5	12.6	6.4
	10				10M	43	29	33.5	7.9	5.8
	15	Stopped	39.3	+	15M	45	30	31.4	8.9	6.8
934	Control		34.6*		Control	41	35	0.1	5.6	3.1
	0	Started 75°								
	25	Stopped	43.5*	+	25M	40	34	31.9	6.5	6.9

* Right heart temperature.

jured red blood cells and that a high erythrocyte content of potassium is essential to its occurrence.

In vitro *effects of heat on pigs' blood:* It was thought that more precise information regarding the reciprocal relationships of temperature, time and the release of potassium from erythrocytes could be obtained by heating samples of pigs' blood *in vitro.*

Heart's blood was collected from normal pigs by cardiac puncture in a heparinized syringe where it was mixed and then discharged into heparinized glass stoppered vials. One vial was kept at room temperature as a control; the others were strapped to a mechanical mixer and immersed in a constant temperature bath. Exposure temperatures ranged between 44° and 63° C. during which time the blood was mechanically decanted from one end of the vial to the other at a rate of 6 times per minute. It required approximately 2 minutes for the temperature of the blood to reach that of the water bath. As soon as a sample was removed from the water bath, it was immediately cooled in ice water and analyzed.

It is apparent that there was a progressive increase in the rate at which potassium passed out of the erythrocytes and into the plasma of the blood as its temperature was raised (Table IV). The amount of the plasma increment at the end of 1 hour's exposure at 40°, 44°, 48°, 51°, 52° and 55° C. were respectively 0.6, 1.6, 6.4, 6.4, 6.7 and 14.3 meq. per L. At the lower temperatures (51° C. and under), the increments were due almost entirely to leakage from intact cells. At the end of 30 minutes exposure at 52° and 55° C., the proportion of the plasma increment contributed by hemolysis was 24 and 38 per cent respectively.

Unequivocal evidence of swelling of erythrocytes was first observed at 55° C. although there

TABLE IV

In vitro *effects of heat on pigs' blood*

Specimen	Temp.	Time	Volume packed cells	Hemoglobin in cells	Hemoglobin in plasma: Hemolysis	Potassium in red cells	Potassium in plasma			
							Total	Change	Increment from hemolysis	Increment from leakage
	° C.	*minutes*		*grams per 100 ml.*	*per cent*	*meq. per L.*	*meq. per L.*			
1–947	Control	Control	30	34	0.1	105	3.2			
	40°	15	30	34	0.1	113*	3.5	+0.3		0.3
		30	30	32	0.3	114*	3.5	+0.3	0.1	0.2
		60	30	34	0.1	102	3.8	+0.6		0.6
2–949	Control	Control	32	33	0	99	3.2			
	44°	15	31	33	0.1	107*	3.9	+0.7		0.7
		30	31	34	0.1	102*	4.0	+0.8		0.8
		60	31	33	0.3	97	4.8	+1.6	0.1	1.5
3–949	Control	Control	31	34	0	104	4.6			
	48°	15	32	31	0.1	101	7.5	+2.9		2.9
		30	32	32	0.1	90	9.2	+4.6		4.6
		60	32	29	0.4	90	11.0	+6.4	0.1	6.3
4–950	Control	Control	33	32	0.0	109	4.3			
	51°	15	35	31	0.8	96	10.2	+5.9	0.4	5.5
		30	34	34	0.5	98	11.8	+7.5	0.2	7.3
		60	36	31	0.7	92	10.7	+6.4	0.4	6.0
5–950	Control	Control	34	35	0.1	120	4.2			
	52°	15	35	34	0.8	103	10.0	+5.8	0.5	5.3
		30	35	32	2.7	101	10.4	+6.2	1.5	4.7
		60	36	32	2.7	100	10.9	+6.7	1.6	5.1
6–947	Control	Control	31	33	0.1	109	4.2			
	55°	15	40	28	1.3	85	7.5	+3.3	0.7	2.6
		30	37	30	5.7	87	12.1	+7.9	3.0	4.9
		60	37	30	9.6	71	18.5	+14.3	4.4	9.9
7–1052	Control	Control	38	33	0.0	119	3.6			
	60°	5	48	28	1.4	83	12.6	+9.0	1.2	7.8
8–1052	Control	Control	36	35	0.1	121	4.1			
	61°	5	45	28	3.5	76	20.8	+16.7	2.4	14.3
9–1052	Control	Control	34	36	0.0	122	3.9			
	62°	5	33	34	11.7	69	30.8	+26.9	4.5	22.4
10–1052	Control	Control	36	36	0.1	121	4.1			
	63°	5	26	34	31.6	58	40.2	+36.1	9.6	26.5

* These values must be due to analytical errors.

may have been some swelling in all specimens exposed for more than 30 minutes at 48° and higher.

The rate of change in the blood was much more rapid during exposures at 60° C. and higher. In these experiments the blood remained in the bath for only 5 minutes and the actual time during which it was at the temperature of the water was approximately 3 minutes. The rises in plasma potassium after such brief periods at 60°, 61°, 62° and 63° were respectively 9.0, 16.7, 26.9 and 36.1 meq. per L. The blood was totally hemolyzed at 65° C.

Not until blood was heated at 60° C. or higher in a test tube were the observed increases in plasma potassium comparable to those that occurred in living pigs after cutaneous exposures at 75° C. This is not to imply that the effects of hyperthermia on blood in a test tube are necessarily similar to those effects in a living animal. Attention has already been called to the fact that asphyxia without rise in temperature may cause hyperpotassemia in a living animal. Although the mean temperature of the blood of a living pig is never raised to 60° C., most or all of its blood

may in the course of its circulation through the over-heated dermis be brought to a much higher temperature than would be recorded by a rectal thermometer or intracardiac thermocouple. It will be recalled from the calculations made in Study I (7) of this series that the superficial portion of the dermis of a living pig reaches a temperature of 60° C. within a second after the surface of the skin has been brought to 75° C. It would appear quite possible then that the temperature of a considerable portion of the blood of an animal that had received an extensive cutaneous exposure to water at 75° C. for as long as 5 minutes would have been raised briefly to the neighborhood of 60° C. during its passage through the superficial subcutaneous tissue.

Not until the temperature of the bath was raised to 62° C. did a 5-minute exposure of blood in a test tube result in hemolysis comparable to that observed in living pigs exposed to 75° C.

Attention has already been directed to the fact that unequivocal swelling of erythrocytes was first observed in a test tube after a 15-minute exposure at 55° C. So far as could be judged by the hemoglobin-hematocrit ratios, swelling of erythrocytes continued through 61° C., beyond which it was not observed.

SUMMARY

These experiments have established that severe and extensive cutaneous burning may result in a rapid rise in plasma potassium to levels ordinarily considered incompatible with life. Such levels are attained when a large proportion of the body surface of an animal whose erythrocytes normally have a high potassium content is maintained at temperatures as high as 75° C. for more than a few minutes. That lower surface temperatures may also be responsible for fatal hyperpotassemia is suggested by the fact that potassium may be released rapidly from blood cells *in vitro* at temperatures of 60° C. Because of the slowness with which potassium is released at lower temperatures and the rapidity with which excess potassium leaves the blood stream, it is not likely that thermal exposures of insufficient intensity to cause severe cutaneous burning could cause sufficient damage to the erythrocytes to produce dangerously high plasma levels.

In vitro experiments on pigs' blood indicate that there is rapid leakage of potassium from intact erythrocytes when the temperature is raised over 60° C. and that rapid hemolysis occurs when the temperature is raised above 62° C. Leakage is accompanied by swelling at temperatures ranging between 55° and 61° C. So far as could be judged by the hemoglobin content of the cells, rapid release of potassium occurs without cell swelling at temperatures above 61° C.

It was demonstrated that leakage from and lysis of red blood cells are the principal sources of the hyperthermic potassium increments of plasma. At the lower temperatures (47° C. *in vivo* and 48° C. *in vitro*) hemolysis is negligible. The increase in plasma potassium *in vivo* at these temperatures is due either to diffusion from extravascular sources or to leakage from erythrocytes. It was obvious that in the *in vitro* exposures at relatively low temperatures leakage from erythrocytes was the only source of the plasma increment. Although theoretically enough potassium could be released by leakage alone to account for potentially fatal plasma levels (in excess of 16 meq. per L.) no such increases were observed without accompanying hemolysis. When blood was heated *in vitro* leakage contributed more than hemolysis to the attainment of such levels. In thermal exposures *in vivo* of sufficient duration and intensity to produce such high levels, hemolysis was the more important causal factor.

BIBLIOGRAPHY

1. Schjerning, Ueber den Tod in Folge von Verbrennung und Verbruhüng vom gerichtsärztlichen Standpunkte. Vierteljahrsschrift fur Gerichtliche Medicine und Öffentliches Sanitätswesen, 1884, 41, 24.
2. Moritz, A. R., Henriques, F. C., Jr., Dutra, F. R., and Weisiger, J. R., Studies of thermal injury. IV. Exploration of casualty-producing attributes of conflagrations. The local and systemic effects of generalized cutaneous exposure to excessive circumambient (air) and circumradiant heat of varying duration and intensity. Arch. Path. In press.
3. Lowry, O. H., and Hastings, A. B., Histochemical changes associated with aging. I. Methods and calculations. J. Biol. Chem., 1942, 143, 257.
4. Bing, F. C., and Baker, R. W., The determination of hemoglobin in minute amounts of blood by Wu's method. J. Biol. Chem., 1931, 92, [illegible].
5. Ham, T. H., Studies on destruction of red blood cells

I. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: an investigation of the mechanism of hemolysis, with observations on five cases. Arch. Int. Med., 1939, 64, 1271.

6. Roos, A., Weisiger, J. R., and Moritz, A. R., Studies of thermal injury. VII. Physiological mechanisms responsible for acute circulatory failure and death during cutaneous exposure to excessive heat. J. Clin. Invest., 1947, 26, 505.

7. Henriques, F. C., Jr., and Moritz, A. R., Studies of thermal injury. I. The conduction of heat to and through the skin and the temperatures attained therein. A theoretical and an experimental investigation. Am. J. Path. In press.

STUDIES OF THERMAL INJURY. VII.[1] PHYSIOLOGICAL MECHANISMS RESPONSIBLE FOR DEATH DURING CUTANEOUS EXPOSURE TO EXCESSIVE HEAT

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INTRODUCTION

In a previous study in this series (1), attention was called to the fact that acute hyperthermic circulatory failure in some animals was accompanied by, and probably contributed to, by large increases in the potassium concentration of the plasma. An investigation of the circumstances in and the sources from which thermally induced rises in plasma potassium occur has been reported (2).

Although it appears that acute potassium poisoning is probably one, it is obvious that it is not the only mechanism by which cutaneous exposure to heat may cause rapidly fatal circulatory failure. The following experiments were undertaken to investigate more fully the physiological mechanisms by which cutaneous hyperthermia may result in acute circulatory failure and death.

The acute physiological disturbances caused by systemic hyperthermia have attracted the attention of a number of investigators. Heymans (3) injected methylene blue into dogs anesthetized with chloralose. This produced a gradually mounting rectal temperature which reached the lethal level of 43.7° C. to 44.8° C. in 1 to 1½ hours. The heart rate rose gradually from 90 to 120 to 300 to 330 per minute. At first, the respirations were deep and rapid (less than 200 per minute); after the temperature had risen to 41.5° to 43.5° C. they became very shallow and even more rapid (over 300 per minute). Systolic pressure rose and diastolic pressure fell. Respiration almost always failed first, and artificial respiration enabled the heart to continue for a longer time. Reflexes persisted up to the time of respiratory standstill. Uyeno (4) produced hyperthermia in cats, anesthetized with urethane, by exposing them to water of 41° to 42° C. or to a high environmental air temperature. During the 30 minutes of exposure the rectal temperature rose from 35° to 39° C. There was little increase in heart rate, but a pronounced rise in minute-volume output. Shortly after exposure the respiratory rate increased to an average of 200 per minute. This breathing was very shallow (tidal air 2 to 3 ml. per minute) and sometimes resulted in a 29 per cent drop in arterial oxygen saturation. Cheer (5) placed dogs anesthetized with morphine and barbital in a cabinet heated by electric light bulbs. In 2 to 3 hours a lethal (rectal?) temperature of 43° to 45° C. was reached. The heart rate increased progressively until a temperature of 42° to 44° C. was reached, when the heart slowed rather suddenly. Before this stage, electrocardiographic abnormalities were limited to slight abbreviation of the PR interval, slight changes in the QRS complex, and inversion of the T wave. The terminal bradycardia was due to the development of nodal rhythm or of various other types of ventricular rhythm. Systolic and diastolic pressures remained fairly constant up to 41° C., then both dropped, the former more than the latter. The respiratory rate also increased. Respiratory standstill usually occurred before cardiac arrest, vagotomy delaying respiratory failure. A progressive decrease of the blood carbon dioxide was found associated with slight alkalosis and rise of oxygen content which were ascribed to the increased pulmonary ventilation. From the same laboratory, Wiggers and Orias (6) reported observations on the effects of short radio waves on dogs. The cardiac acceleration, increase in rate and depth of the respiration, and primary failure of the respiration were identical with the findings of Cheer (5). However, instead of a decrease in blood pressure, a rise of systolic and diastolic pressure was observed which progressed until death.

Clinical observations on the effect of hyper-

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thermia were made by Ferris (7) *et al.* Patients with heat stroke whose rectal temperatures varied from 39.9° to 44.0° C. exhibited a hot dry skin, a normal or elevated systolic pressure, which dropped to low levels only in the terminal stage, and venous pressures of from 2 to 12 cm. of saline. Their respiratory rate was 28 to 50 per minute. Of 29 patients (all comatose) whose temperatures exceeded 41.5° C., 17 died; all others recovered.

Attempts to analyze the disturbances observed in the intact organism by elevating the temperature in one organ have been made since 1872. Fick (8) heated the blood as it passed through the carotid arteries of the dog and noticed marked hyperpnea without change in heart rate or blood pressure. Cyon (9) isolated the circulation of a dog's head. Perfusion of the head with heated blood produced bradycardia and a drop in blood pressure. Kahn (10) warmed the carotid arteries of unanesthetized dogs without producing a rise in rectal temperature. He observed the development of tachycardia and a moderate rise in blood pressure. Moorhouse (11) heated the carotids and simultaneously cooled the jugular veins in dogs. This resulted in tachycardia, rarely preceded by bradycardia, ascribed respectively to increased sympathetic and vagal activity. Coincidentally, tachypnea and peripheral vasodilatation were observed. Heymans and Ladon (12) severed all connections except the vagal nerves between head and trunk of dogs anesthetized with chloralose. Artificial respiration was applied and the circulation in the head maintained by connecting it to a donor dog. The sublingual temperature of the preparation rose to 45° C. in 1½ hours. There was no change in the heart rate which had risen to 160 after severance of the cervical cord. The head exhibited a progressive and pronounced increase in respiratory rate which persisted until a sublingual temperature of 45° C. was reached when the rate rapidly decreased and the reflexes of the head, which had been active up to that time, disappeared.

The effect of hyperthermia on the heart was investigated by Knowlton and Starling (13) using the innervated heart-lung preparation, perfused with heated blood. From 26° C. to approximately 45° C. the heart rate was a linear function of the blood temperature, the rate at 45° C. being 180 per minute. Above this temperature marked slowing occurred and the heart soon stopped. Arrhythmias occurred above 40° C.

Summarizing these data, it can be said that in the dog, the highest rectal temperature compatible with life lies between 43° and 45° C., when this temperature is reached in 1 to 3 hours. Respiratory failure often seems to precede circulatory failure. Tachypnea, tachycardia, and peripheral vasodilatation seem to be, in part at least, of cerebral origin.

The physiological changes of rapidly developing hyperthermia leading to death within ½ hour have not been heretofore studied. As high environmental temperatures are needed for such experiments, the results are necessarily complicated by the damaging effect of heat on the skin directly. Moreover, these high temperatures will produce damage to the red blood cells that are circulating in the small vessels of skin and underlying tissues (Shen *et al*, 14).

EXPERIMENTAL PROCEDURE

Young pigs, weighing from 6.4 to 18 kgm., and adult dogs, weighing from 7.4 to 8.5 kgm., were used as experimental animals. They were anesthetized with pentobarbital sodium (32 mgm. per kgm. intraperitoneally), shaved, and tied to a wooden animal board. This was lowered into a galvanized iron tank (92 × 46 × 41 cm.). The head of the board rested on a metal bar in the tank, so that it was slightly higher than the foot. A similar tank, placed on a high table, partly projected over the former. This tank was filled with water steam-heated to the desired temperature. In the bottom of the projecting part was a circular opening, 13 cm. in diameter, that could be closed with a heavy rubber and metal stopper. Removal of this stopper resulted in immersion of more than ½ the body surface of the animal in 8 to 10 seconds. During immersion, the temperature of the water, which was continuously stirred, was kept within narrow limits by intermittent introduction of steam. Drainage of the water and termination of exposure could also be accomplished in 8 to 10 seconds. Temperatures ranging from 44° to 75° C. were used.

Previous to exposure, all animals were heparinized (3 mgm. per kgm. intravenously). Because of spasmodic closure of the glottis on immersion, a tracheal cannula was inserted. The carotid pressure was recorded with a mercury manometer. The right auricular pressure was measured by means of a rubber catheter introduced into the superior vena cava or right auricle by way of the external jugular vein and connected with a water manometer. The level of the right auricle as determined by opening the chest at the end of the experiment was taken as point of reference. In pigs the hydrostatic pressure did not influence the auricular pressure. In dogs immersion resulted

in a considerable rise in recorded auricular pressure, so that only changes occurring during exposure could be compared. Pneumograms were obtained by means of a copper cannula thrust between the ribs into the pleural space and connected by means of a rubber tube to a writing tambour. In other experiments, a tracheal cannula provided with a sealed-in side tube connected to the tambour was used.

Electrocardiograms were taken with an amplifier type of electrocardiograph. It was only possible to take the first standard lead, as the hind legs of the animal were under water. In some experiments, curarized animals were used and artificial respiration was applied throughout the exposure. Intocostrin (Squibb), 1 mgm. per kgm. diluted with saline, was slowly injected intravenously. The side reactions were limited to a short (20 to 30 seconds) period of mild excitation. The drug had no effect on the arterial pressure. A second smaller dose usually had to be given 20 to 40 minutes later. A Palmer respiration pump for small animals which allows the air to escape spontaneously on expiration was used. When venous pressures were recorded the animals were immersed in such a man-

TABLE I

Rectal temperature, arterial pressure and electrocardiogram of 12 pigs immersed in hot water

A—Normal sinus rhythm (normal rate, tachycardia or bradycardia). Normal duration of QRS complex.
A′—First or second degree A-V block. Normal duration of QRS complex.
A″—Complete A-V block. Normal duration of QRS complex.
B—Slight, BB—Moderate, BBB—Pronounced: Widening of QRS complex without P wave.
BBB—Can often be interpreted as ventricular fibrillation.

Time (min.)	Time (sec.)	Rectal temp. (° C.)	Arterial pressure (mm. Hg)	ECG
Pig 876 (7.7 kgm.) 48° C. Died after 26.5 min.				
Control		34.3	118	A
16	00	44.0	66	A
24	30	45.2	42	A
26	30	45.7	76	BB
Pig 875 (6.4 kgm.) 48–50° C. Died after 35 min.				
Control		35.0	130	A
27	30	42.2	64	A
29	00	42.8	64	A
34	15	43.7	26	A
Pig 878 (12.0 kgm.) 47° C. Died after 50 min.				
Control			110	A
29	00		70	A
37	20		50	A
49	30	44.9	30	A
Pig 879 (11.8 kgm.) 44–47° C. Died after 106.5 min.				
Control		36.8	106	A
33	00	43.1	54	A
Out of hot bath* from 33.5 to 48.5 min.				
49	53	42.0	116	
70	30	44.1	86	A
105	00	44.5	14	A

Time (min.)	Time (sec.)	Rectal temp. (° C.)	Arterial pressure (mm. Hg)	ECG
Pig 895 (18.0 kgm.) 49° C. Curare. Died after 32 min.				
Control		37.8	148	A
15	00	41.9	172	A
25	30	43.7	76	A
31	30	44.0	10	A′**
Pig 943 (8.3 kgm.) 47° C. Curare. Died after 36 min.				
Control		37.7	126	A
17	00	42.6	126	A
29	00	44.5	136	A
Pig 897 (16.4 kgm.) 47° C. Curare. Died after 56 min.				
Control		37.9	146	
24	00	43.5	146	A
47	00	44.0	90	A
55	00		36	A′
Pig 946 (9.5 kgm.) 47° C. for 23 min. Curare. Died after 42 min.				
Control		40.1	82	A
17	00	43.0	120	A
26	00	44.4	40	A
34	30	44.6	26	A

Time (min.)	Time (sec.)	Rectal temp. (° C.)	Arterial pressure (mm. Hg)	ECG
Pig 944 (10.4 kgm.) 47° C. for 25 min. Died after 99 min.				
Control		38.1	108	A
14	00	43.5	120	A
26	00	45.4	100	A
37	00	44.1	90	A
Pig 867 (7.3 kgm.) 64–65° C. Died after 15 min.				
Control			146	A
5	30		72	A
10	30		72	A
15	00	46.0	12	BB
Pig 872 (7.3 kgm.) 64–65° C. Died after 11 min.				
Control			150	A
7	00		50	A
10	30		50	BB
10	45		40	BBB
Pig 871 (9.1 kgm.) 70–73° C. Died after 12 min.				
Control			100	
5	30		74	A
6	10		74	BB
9	30		[illegible]	BBB
12	00	44.5	[illegible]	BBB

* Skin temperature lowered by exposure to cool water between two [illegible]
** Occasional ventricular extrasystole.

ner that most of the anterior thorax remained above the water level. This was sufficient to abolish artefacts produced by the increased resistance to the inflow of air.

Temperatures were recorded with a thermocouple introduced to a depth of 7 to 9 cm. into the rectum, which had been cleaned by repeated enemas. The anus was closed around the couple. In 3 experiments, heart temperatures were also recorded by means of a thermocouple introduced through the external jugular vein into the right auricle. In some experiments only initial and final rectal and final heart temperatures were measured with a sensitive thermometer. In a considerable number of animals blood was withdrawn from the jugular vein both before and during exposure for the determination of the hematocrit and of hemoglobin and potassium content of red cells and plasma. In most instances, immersion was continued until death. In some experiments exposure was temporarily interrupted, and in a few cases, immersion was terminated at a time when the animal was still living.

In addition to these observations, 3 pigs were infused with an isotonic (1.12 per cent) solution of KCl. Frequent electrocardiograms (lead I or II) were taken. In one of these pigs, the arterial and right auricular pressure and respirations were also recorded. The latter ani-

TABLE II

Rectal temperature, arterial pressure, electrocardiogram, and potassium content of plasma of 15 pigs immersed in hot water

A—Normal sinus rhythm (normal rate, tachycardia or bradycardia). Normal duration of QRS complex.
A′—First or second degree A-V block. Normal duration of QRS complex.
A″—Complete A-V block. Normal duration of QRS complex.
B—Slight
BB—Moderate
BBB—Pronounced
} Widening of QRS complex without P wave.
BBB—Can often be interpreted as ventricular fibrillation.

Time (min.)	Time (sec.)	Rectal temp. (° C.)	Arterial pressure	ECG	K plasma (meq. per L.)
Pig 877 (7.0 kgm.) 47° C. Died after 26 min.					
Control		34.3	96	A	3.8
10	20	41.6	136	A	6.2
14	05	42.5	112	A″	6.9
24	10	44.3	56	A″	8.2
Pig 923 (13.6 kgm.) 47° C. Died after 50 min.					
Control			116	A	3.8
13	15		146	A	5.5
22	30		148	A	5.5
34	15		102	A	6.2
42	00		56	A	6.5
46	33		66	A	7.5
Pig 1057 (8.0 kgm.) 47° C. Died after 36.5 min.					
Control		37.0		A	4.4
19	50			A	7.0
36	15			A	10.2
36	30	45.5		0	
Pig 1056 (7.0 kgm.) 47° C. Died after 44.5 min.					
Control		37.8		A	4.7
9	30			A	5.9
15	07			A	7.2
34	00			A	7.1
44	30	45.5		0	

Time (min.)	Time (sec.)	Rectal temp. (° C.)	Arterial pressure	ECG	K plasma (meq. per L.)
Pig 910 (9.5 kgm.) 72–75° C. Died after 12.5 min.					
Control		36.8	148	A	3.0
2	15	40.7	100	A	19.1
4	40	40.7	86	BB	18.1
7	20	41.5	74	BBB	24.0
13	52	43.7	10	0	17.3
Pig 912 (10.0 kgm.) 72–75° C. Died after 14 min.					
Control		36.0	88	A	4.1
1	20	35.4	154	A	16.7
3	35	37.0	98	BB	
5	07	37.1	74	BB	16.4
9	45	40.8	74	BBB	16.4
13	40	43.1	30	BBB	
Pig 908 (9.1 kgm.) 75° C. Died after 13.5 min.					
Control			96	A	3.8
3	40		96	BB	16.7
8	55		60	BBB	18.5
11	10		52	BBB	17.1
Pig 907 (10.4 kgm.) 75° C. Died after 10 min.					
Control		37.1	116	A	3.5
		37.3*			
6	00	39.0	48	BBB	
		42.7*			
7	30	39.2	32	BBB	17.4
		42.5*			

* Right heart temperature.

TABLE II—*Continued*

Time (min.)	Time (sec.)	Rectal temp. (° C.)	Arterial pressure	ECG	K plasma (meq. per L.)
Pig 905 (12.7 kgm.) 75° C. Curare. Died after 23 min.					
Control			94	A	4.8
16	30	41.6	78	BB	
22	40	42.1	32	BBB	17.3
Pig 921 (16.8 kgm.) 75° C. Curare. Died after 27 min.					
Control			122	A	3.2
3	30		66	A	5.1
8	00		58	BB	11.6
18	00		36	B	11.9
26	45		28	B	10.2
Pig 906 (13.0 kgm.) 70–75° C. Curare. Died after 70 min.					
Control		38.6	102	A	4.0
10	50	41.4	112	BBB	
16	35	42.3	62	BB	17.4
25	20	43.0	92	BBB	15.2
44	35	44.6	72	BB	13.3
46	40	44.8	46	A	
48	29	45.0	46	BB	
65	00	46.8	46	BBB	
Pig 913 (8.2 kgm.) 75° C. for 6.5 min. Died after 7.5 min.					
Control		38.6	100	A	3.5
2	25	37.9	100	B	14.2
6	15	40.5	50	BBB	17.7
7	45	40.8	15	0	17.4

Time (min.)	Time (sec.)	Rectal temp. (° C.)	Arterial pressure	ECG	K plasma (meq. per L.)
Pig 919 (9.1 kgm.) 75° C. for 5 min. Died after 18 min.					
Control		37.1	138	A	4.2
4	15	41.1	78	BB	25.5
7	45	42.3	28	A	21.4
10	10	43.2	26	A	18.3
14	00	44.2	30	B	17.0
16	45	44.3	14	B	17.5
Pig 918 (8.7 kgm.) 75° C. for 3 min. Died after 55 min.					
Control		36.6	70	A	3.7
4	25	38.7	56	A	11.0
11	00	39.7	62	A	9.5
17	05	40.3	70	A	9.5
37	00	40.6	70	A	9.4
Pig 899 (13.6 kgm.) 75° C. for 1 min. Sacrificed after 77 min.					
Control		37.4	142	A	3.6
5	15	40.5	30	A	10.2
16	05	40.5	76	A	6.9
45	45	40.3	76	A	4.2
76	00	39.2	76	A	7.4

mal received the solution in the subclavian vein, the other two in the jugular vein. Blood samples for the determination of potassium were taken from the carotid artery.

RESULTS OF EXPERIMENTS

In Table I are shown the results of 12 experiments in which pigs were exposed for varying periods of time at temperatures ranging between 44° and 73° C. Changes in rectal temperature, arterial pressure, and electrocardiogram are indicated.

In Table II are shown the results of 15 experiments in which pigs were exposed at temperatures ranging between 47° and 75° C. The changes that occurred in the potassium concentration of the plasma are indicated in relation to changes in rectal temperature, arterial pressure, and electrocardiogram.

In Table III are shown the results of 5 experiments in which dogs were exposed for varying periods of time at temperatures ranging between 55° and 75° C. The changes that occurred in the potassium concentration of the plasma are indicated in relation to changes in rectal temperature, arterial pressure, and electrocardiogram.

In Table IV are shown the results of 3 experiments in which pigs received intravenous infusions of isotonic potassium chloride. The changes in the potassium concentration of the plasma and the erythrocytes are indicated in relation to changes in hematocrit, arterial pressure, and electrocardiogram.

Arterial blood pressure. The immediate effect of immersion in water of [illegible] to 75° C. upon the mean arterial pressure of pigs [illegible] a [illegible]

TABLE III

Rectal temperature, arterial pressure, electrocardiogram, and potassium content of plasma of 5 dogs immersed in hot water

A—Normal sinus rhythm (normal rate, tachycardia, or bradycardia). Normal duration of QRS complex.
B—Slight widening of QRS complex without P wave.

Time		Rectal temp.	Arterial pressure	ECG	K plasma
min.	*sec.*	° *C.*	*mm. Hg*		*meq. per L.*
Dog 931 (7.4 kgm.) 55° C. Died after 23 min.					
Control		35.4	112	A	2.8
5	10	37.0	92	A	5.2
13	15	40.6	58	A	4.7
20	45	41.4	18	A	6.9
Dog 930 (7.5 kgm.) 60° C. Died after 16.5 min.					
Control		36.9	100	A	4.0
4	45	37.4	86	A	3.3
7	55	38.0	64	A	4.7
10	40	39.1	66	A	5.3
Dog 922 (8.5 kgm.) 75° C. Died after 15 min.					
Control		37.9	118	A	3.1
2	55	37.6	90	A	5.8
6	30	38.4	68	A	6.4
10	20	39.0	76	A	5.8
15	00	39.3	30	A	6.8
Dog 929 (8.2 kgm.) 75° C. Died after 13.5 min.					
Control		37.2	130	A	3.9
3	10	38.5	130	A	4.8
8	30	42.1	120	A	6.1
12	45	44.1	74	B	8.2
Dog 934 (7.6 kgm.) 75° C. Died after 25 min.					
Control		34.6*	148	A	3.1
15	16	41.7*	100	A	6.9
24	45	43.5*	72	A	

* Right heart temperature.

sometimes amounted to as much as 140 mm. Hg. This rise also occurred in curarized animals or when hot water was splashed on the skin. It was absent at immersion temperatures of 45° to 47° C.

At temperatures of 44° to 59° C. the blood pressure was maintained at or above pre-immersion level for 16 to 26 minutes. It began to fall at variable times during exposure and reached half of the original value in 17.5 to 41 minutes. The rectal temperature at this time had risen from 34.3° to 40.1° C. to 42° to 44° C. These animals

TABLE IV.

Physiological and chemical changes in three pigs intravenously infused with an isotonic (1.12 per cent) solution of KCL

A—Normal sinus rhythm (normal rate, tachycardia or bradycardia). Normal duration of QRS complex.
B—Slight
BB—Moderate
BBB—Pronounced } Widening of QRS complex without P wave.
BBB—Can often be interpreted as ventricular fibrillation.

Time		Arterial pressure	ECG	Hematocrit	K plasma	K cells
min.	*sec.*	*mm. Hg*			*meq. per L.*	*meq. per L.*
Pig 901 (14.8 kgm.). Rate of infusion *0.6 ml. per kgm. per min.* Died after 50 min.						
Control			A[1] (lead I)	36	4.3	123
11	00		A[1]	36	9.0	125
16	00		A[1]	37	9.5	124
18	00		BB	38	11.2	121
26	00		BBB	37	15.5	132
		Infusion stopped				
26	10		0			
35	00		0			
36	00		A			
41	00		A	38	8.7	139
41	30	Infusion started again. Rate *0.7 ml. per kgm. per min.*				
50	00		BBB	35	17.7	136
Pig 911 (8.7 kgm.). Rate of infusion *0.9 ml. per kgm. per min.* Died after 22.5 min.						
Control			A (lead II)	35	3.2	127
11	00		A[2]	34	8.7	122
14	00		B	35	10.6	122
16	00		BBB	35	12.7	125
20	00		BBB	31	27.0	
22	00		0	28	38.0	127
Pig 925 (15.9 kgm.). Rate of infusion *0.6 ml. per kgm. per min.* Died after 39 min.						
Control		76	A (lead II)	33	3.5	112
6	08	76	A	33	5.7	117
12	40	76	A	32	10.6	114
19	37	76	A[3]	34	12.7	110
24	50	76	B	37	15.7	109
35	18	24	BBB	37	26.1	111

1—P wave not clearly shown.
2—P wave getting blunt.
3—P wave very flat.

died after 25.5 to 50 minutes with rectal temperatures of 43.9° to 45.8° C., the heavier pigs surviving somewhat longer than the lighter ones. Heart temperatures were within a few tenths of a degree of these values (Figure 1).

In pigs exposed to water of 60° to 75° C., the arterial pressure was maintained for 1 to 6 minutes, and reached half of its original value in 5.5 to 11 minutes. The animals died after 8 to 15 minutes with rectal temperatures varying from 39.4° to 46.0° C. However, the discrepancy between heart and rectal temperature often was considerable (Figure 1).

The possible reversibility of the fall in arterial pressure was investigated. Immersion of a pig at 47° C. for 33 minutes produced a fall in blood pressure from 104 to 40 mm. Hg (Figure 2). Exposure to cool water brought the pressure back to its original level and lowered the rectal temperature from 43.3° to 42.0° C. Re-exposure to 47° C. again resulted in a fall in blood pressure, and death occurred at a rectal temperature of 44.5° C. In another instance exposure to water of 75° C. for 1 minute reduced the pressure from 140 to 20 mm. Hg in 5 minutes. During subsequent exposure to room air the pressure recovered, and reached 130 mm. Hg after 73 minutes. The animal was still alive after more than 2 hours. Exposure of one animal to water of 75° C. for 5 minutes resulted in a fall in blood pressure from 138 to 75 immediately after immersion. The pressure continued to fall, and the animal died after 18 minutes.

The arterial pressure in dogs behaved in a way comparable to that in pigs at the same temperature. Animals immersed at 60° to 75° C. survived for 13.5 to 25 minutes (Table III).

Right auricular pressure: Intra-auricular pressures of pigs before immersion varied from 32 to − 66 mm. H_2O (average − 23 mm. H_2O). In only 3 out of 15 animals was the pressure in the right auricle higher than atmospheric (+ 13, 20, and 32 mm. H_2O). In most instances, a slight rise occurred following immersion, the control level

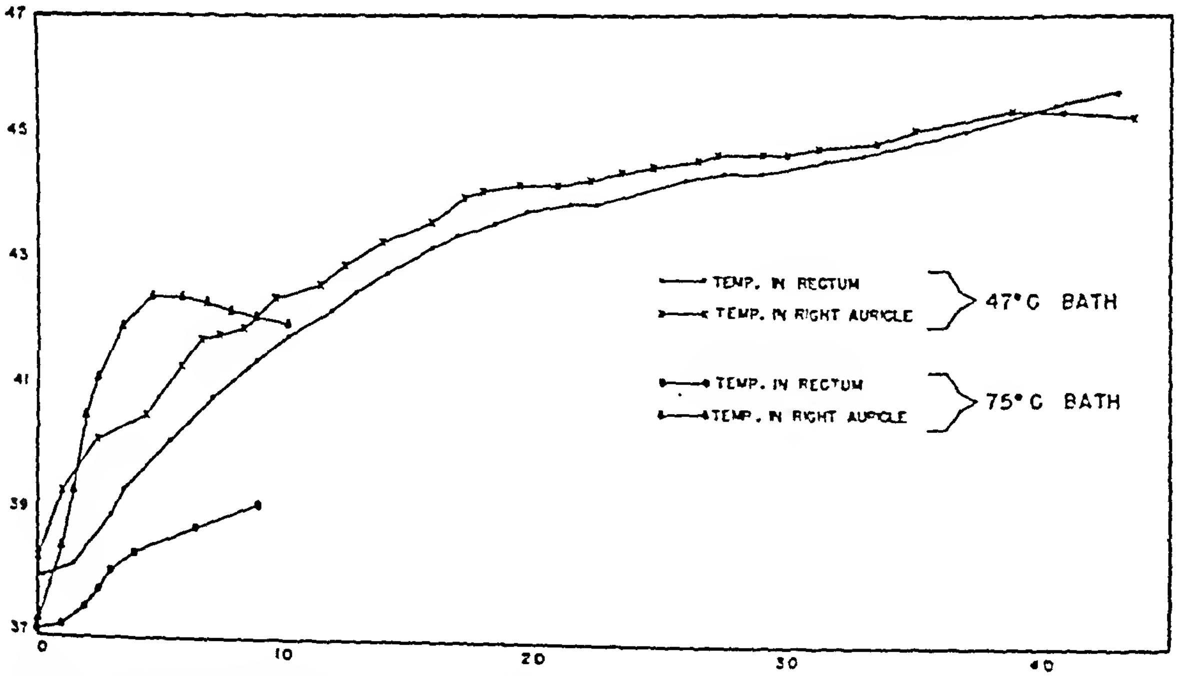

FIG. 1. PLOT OF THERMOCOUPLE RECORDINGS SHOWING RATE OF CHANGE IN RECTAL AND RIGHT [illegible] BLOOD TEMPERATURES DURING IMMERSION IN LOW (47° C.) AND HIGH (75° C.) TEMPERATURE [illegible]

47° C.—Pig No. 882 (13.2 kgm.)

75° C.—Pig No. [illegible] (10.5 kgm.)

It may be seen that although the right auricular blood temperature rises rapidly after [illegible] considerable lag in the temperature rise in the rectum. The higher the temperature of the bath [illegible]

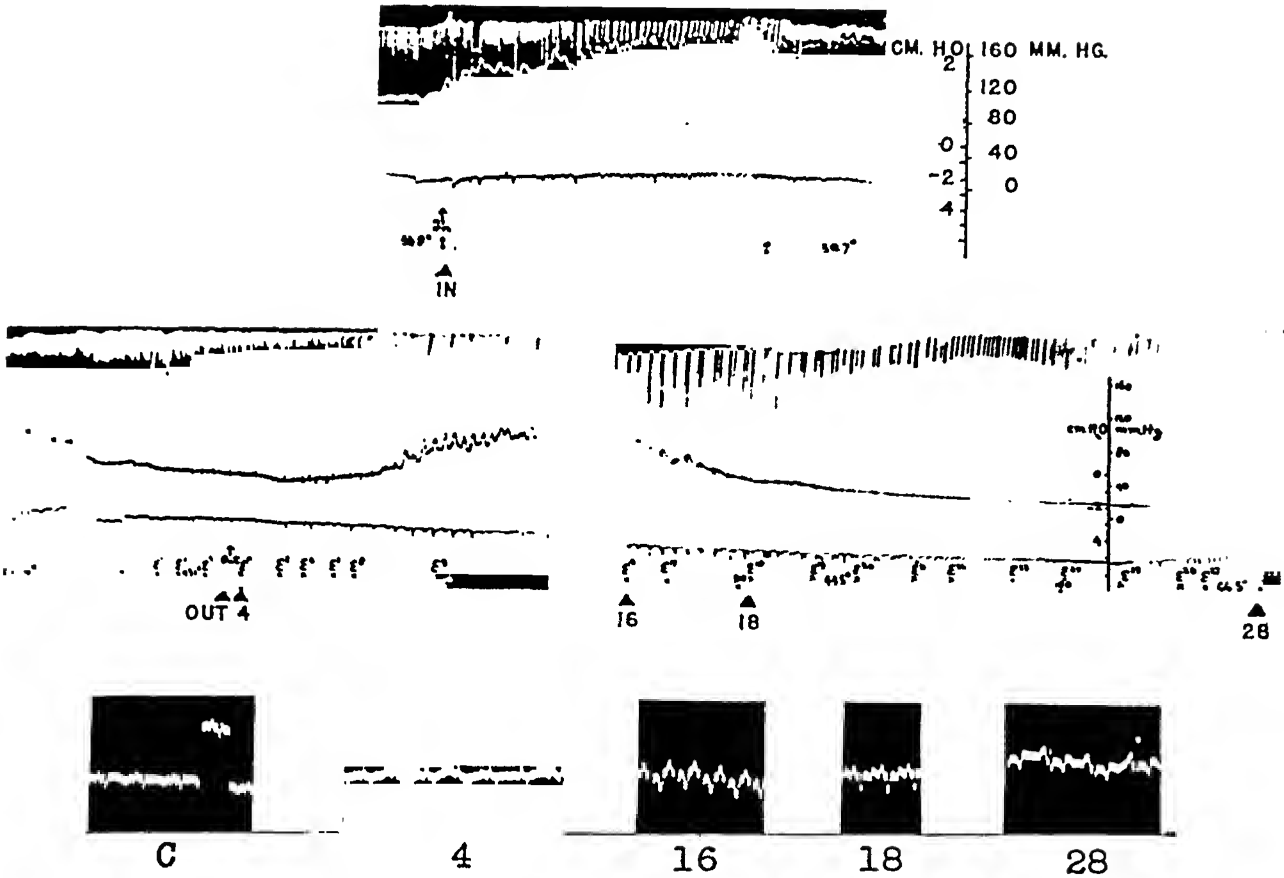

FIG. 2. EFFECT OF 2 EPISODES OF CUTANEOUS HYPERTHERMIA ON PIG NO. 879 (11.8 KGM.) CAUSED BY IMMERSION IN WATER AT 47° C.

The first period of immersion lasted for 33.5 minutes and is indicated by the words "in" and "out" on the first and second segments of the kymograph record. Fifteen minutes after the end of the first period of hot water immersion and between the second and third segments of the record the animal was immersed again at 47° C. and allowed to remain in the bath until dead (56.5 minutes). Between the 2 episodes of hot water immersion the skin temperature was lowered by exposure to cool water. Total duration of experiment was 105 minutes. The upper, middle and lower tracings on the kymograph record represent respectively the pneumogram, the carotid pressure, and the right auricular pressure. The times at which the electrocardiograms were taken are indicated in minutes.

being regained in 0.5 to 3 minutes. In 5 of the 6 animals immersed at 44° to 49° C., this was followed by a gradual drop of 4 to 20 mm. H_2O. There was no rise in venous pressure until 1 or 2 minutes before death. In the sixth pig, immersion did not influence the auricular pressure (Figure 2).

In 7 of the 9 pigs exposed to water of 60° to 75° C., a gradual rise of the right auricular pressure was observed, beginning in the middle of, or even early in exposure, and continuing until death. This rise amounted to 15 to 45 mm. H_2O and occurred at a time when both arterial pressure and respiration were still adequate (Figure 3). In some instances, it was preceded by a fall of 20 to 30 mm. H_2O which rapidly developed 1 to 3 minutes after the exposure had started. In 2 animals, this fall was the only change in auricular pressure that was observed until 1 minute before death when it rapidly rose.

One pig, exposed for only 1 minute to water of 75° C., showed an abrupt fall of 40 mm. H_2O. During the following 70 minutes the auricular pressure gradually returned to the pre-immersion level, coincidentally with recovery of the arterial pressure.

The auricular pressure of 4 dogs was lower than that of the pigs. It ranged from − 77 to − 108 mm. H_2O. Because of hydrostatic effects the auricular pressures before and during immersion could not be compared. However, neither in the 2 dogs exposed to 75° C. nor in those exposed to

55° and 60° C. was there observed any change in the recorded auricular pressure during the period of immersion.

Because of the possible contributions of the type or rate of breathing to the observed pressure changes, some experiments were performed on curarized pigs. Artificial respiration was supplied throughout the experiments. The course of the auricular pressure was found to be identical with that of the spontaneously breathing animals. At 47° to 49° C. a slow and moderate fall was observed; exposure at 75° C. resulted in a rise, beginning early during exposure.

Respiration: In agreement with earlier investigators, it was found that a rise in body temperature was associated with a pronounced increase in respiratory rate. In the pig, the immediate effect of immersion was usually a short period of very deep and fairly rapid respirations, followed by a variable episode of only moderately increased breathing (rate 20 to 40). In the animals exposed to the lower temperature range the onset of respiratory rates of 170 to 200 was often sudden, and occurred in the first 10 minutes of exposure, at rectal temperatures of 39° to 41° C. Deep gasps interrupted this shallow tachypnea. The arterial blood maintained its bright red color. The tachypnea gradually increased, and rates of 300 were not infrequently reached. When the rectal temperature had mounted to 43° to 44° C., breathing abruptly slowed to 10 to 40 per minute and became much deeper. Additional slowing usually continued until death. In the dog, immersion was immediately followed by a tachypnea of 100 to 150 per minute, which gradually increased. Rates over 200 were not encountered.

It is difficult to estimate whether the respiratory or the circulatory system failed first in these animals. If bradypnea is considered as the first manifestation of failing respiration it might be said that the cardiovascular system survived somewhat longer, as judged by the presence of an appreciable arterial blood pressure. However, at least in the beginning of bradypnea, the pulmonary ventilation certainly was as adequate as during the control period. If the onset of prolonged apnea is considered as the end point of adequate respiratory function, both systems failed simultaneously. In 3

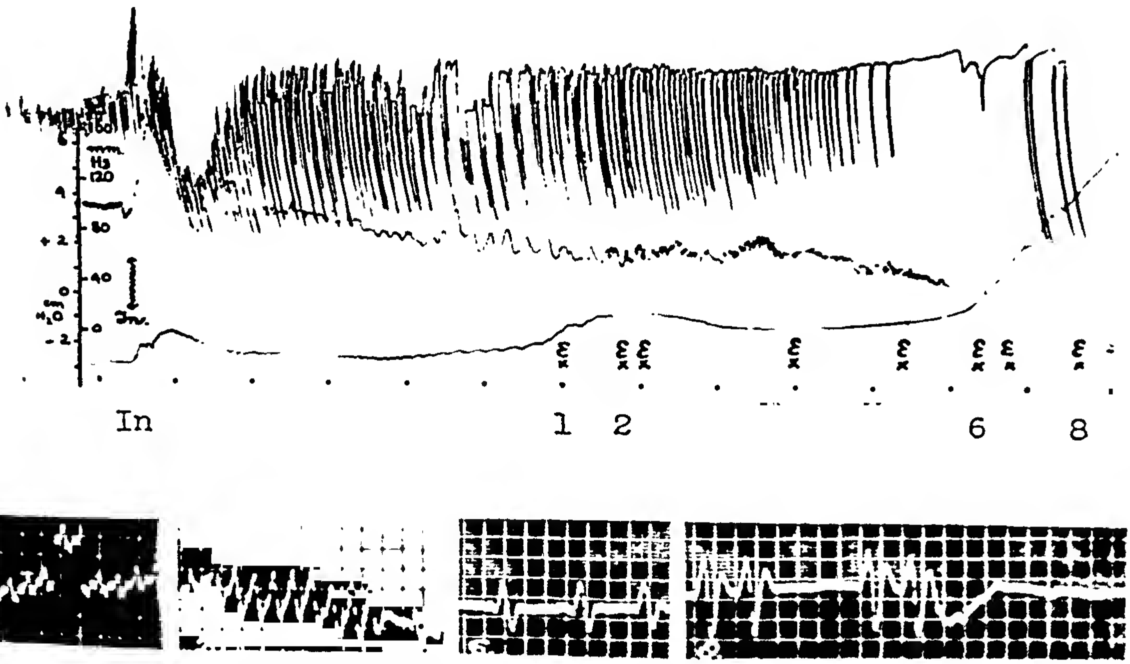

FIG. 3. EFFECT ON PIG NO. 871 (9 [illegible] KGM.) OF IMMERSION IN WATER BATH AT 70° TO 73° C. [illegible] 12 [illegible]

The upper, middle and lower tracings on the kymograph record represent respectively the [illegible] pressure and the right auricular pressure. The sequence in which the electrocardiograms were taken [illegible]

animals, artificial respiration was applied at a time when the arterial pressure was still appreciable (80–90 mm. Hg), without having the slightest effect upon its downward course. Moreover, the final rectal and heart temperatures of the curarized pigs fell well within the range of those of spontaneously breathing animals.

Exposure of pigs to 60° to 75° C. produced an increase in respiratory rate which did not exceed 80 to 90 per minute. The breathing remained deep until the terminal episode of bradypnea, ending in occasional deep gasps. In dogs the respiratory changes were essentially the same as those encountered at the lower temperatures.

Electrocardiographic changes: In both pigs and dogs, the first change, beginning immediately after immersion, consisted of a progressive increase in heart rate to levels of 300 to 350 per minute. Associated with this increase, changes occurred in the QRS complex, consisting of decrease in amplitude of the R wave and deepening of the S wave, or vice versa, with maintenance of the normal QRS interval and inversion of the T wave. The changes in the initial ventricular deflection might, in part at least, be due to variations in type of breathing with resulting changes in the position of the heart (Harris (15)). They occurred only to a minor degree in curarized animals.

In the pig, the abnormalities following this sinus tachycardia varied markedly with the temperature of exposure. Of all animals exposed to water at 44° to 50° C. (Tables I and II) only one showed appreciable widening of the QRS complex and loss of P wave. This occurred 1 minute before death. Another animal showed disappearance of the P waves. The changes in the remaining pigs were limited to sinus bradycardia and sinus arrhythmia that became most pronounced 2 or 3 minutes before death (Figure 2). Occasionally, auriculoventricular block of varying degree was seen during this period.

In contrast, 11 pigs continuously exposed to temperatures of 64° to 75° C. (Tables I and II) all showed the gradual development of exceedingly wide ventricular complexes with very large T waves, and the gradual disappearance of the P wave.[2] The general shape of these complexes resembled that of the original supraventricular ones. Their development was usually associated with definite slowing, although the heart rate remained regular. In some cases, the transitional phase consisted of salvos of fairly rapid and wide ventricular complexes, which interrupted a still-existent sinus bradycardia. In the terminal stage, the initial ventricular deflection could not be separated from the final one. The electrocardiogram consisted either of very slow, extremely wide ventricular waves, separated from each other by isoelectric intervals of 0.2 to 1.0 second, or of more rapid variations at 160 to 240 per minute, in which one wave merged with the next. The latter state might be called ventricular fibrillation (Figures 3 and 4).

In 9 of the 11 pigs, these changes made their first appearance early during immersion, at rectal temperatures of 37.0° to 41.6° C., and at a time when the arterial pressure and respiration were still adequate. In four of these, the blood pressure at the time of onset of the wide complexes was actually equal to, or higher than, that before immersion. In only 2 animals were the abnormalities first noticed when the pressure had fallen to low levels, and it is possible that they would have been demonstrated earlier if more electrocardiograms had been taken. Exposure for 6.5 and 5 minutes similarly resulted in marked widening of the QRS complex, whereas exposure for 3 and 1 minutes did not produce deviations other than those at lower temperatures.

In the dog, the electrocardiographic changes at high temperatures were in no way different from those encountered at 44° to 50° C. (Table III). They were limited to an increase in rate and to minor changes in the ventricular complex. No widening occurred and the auricular manifestations remained present until the end.

Chemical changes: A complete discussion of the effect of temperature on the potassium concentration of the plasma has been reported (2). The potassium concentration of the plasma of 15 pigs in which physiological studies were made is shown in Table II. The initial plasma levels ranged be-

[2] During tachycardia, actual observation of this disappearance was impossible because of overlapping of P and preceding T waves. In these instances, it was assumed that the same changes had taken place as in the instances where the P wave could be followed through a stage of decreasing amplitude to disappearance, as subsequent slowing of the beat similarly revealed the absence of auricular complexes.

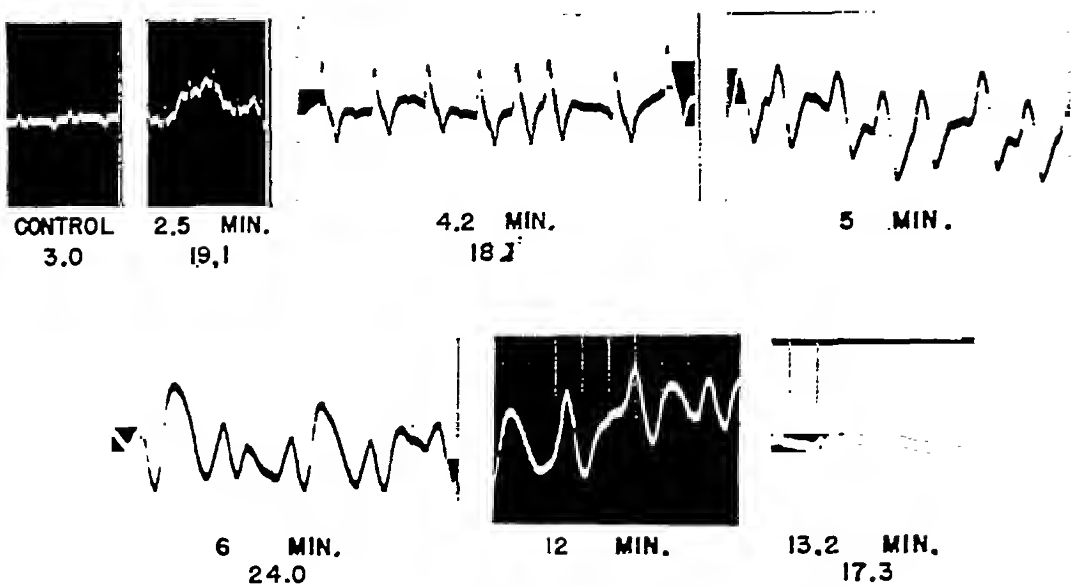

FIG. 4. RELATIONSHIP BETWEEN PLASMA POTASSIUM LEVEL AND CHANGES IN ELECTROCARDIOGRAM (LEAD 1) DURING IMMERSION OF PIG NO. 910 (9.5 KGM.) IN WATER BATH AT 72° TO 75° C.

The plasma potassium values are given in meq. per L. Death occurred 12.5 minutes after beginning of experiment.

tween 3.0 and 4.8 meq. per L. The potassium concentration of the red blood cells ranged from 113 to 145 meq. per L. The course of these concentrations during immersion varied markedly with the temperature.

Immersion of 4 pigs at 47° C. produced a gradual and sustained rise in plasma potassium. Ten minutes exposure resulted in levels of about 6.0 meq. per L. During the rest of the exposure, the level increased by an additional 1 to 4 meq. The highest level was 10.2 meq. per L. obtained 30 seconds before death.

On the other hand, continuous exposure at 70° to 75° C. characteristically resulted in an enormous rise in the plasma potassium level. This increase was found to take place with surprising rapidity. In 5 pigs, the plasma after 1 to 4 minutes of exposure, contained 14.2 to 25.5 meq. per L. of potassium. A sample drawn in this period from one curarized pig was still essentially normal and the peak observed in this animal was only 11.9 meq. Peaks from 16.7 to 25.5 meq. were observed in 6 pigs during exposure. Curare did not prevent rises in this range in 2 pigs; however, no early observations were made on these animals. In some instances, the potassium level fell towards the end. However, it remained markedly elevated.

In some experiments the exposure was terminated before the animal had expired. Immersion for 6.5 and 5 minutes similarly resulted in a tremendous rise of plasma potassium. At the time of death, the level was still very high. Immersion for 3 and 1 minutes produced a less pronounced increase; at the time of death, the level was only 2 to 2.5 times the normal one.

DISCUSSION

These observations show that the physiological disturbances leading to death in pigs exposed to water at 46° to 50° C. are of a different nature from those encountered in animals exposed to temperatures of 60° to 75° C.

In pigs immersed at the lower temperatures, the occurrence of a gradual fall in right auricular pressure followed by a fall in mean arterial pressure, indicates a progressive decrease in venous return to the heart. That this decrease, at least during a major part of the exposure, was due to an increase in capacity of the peripheral vascular bed, rather than to loss of intravascular fluid, is evident from the fact that the changes in circulatory dynamics were found to be reversible to a considerable degree. As the exposure continued the detrimental effects of the heated blood [illegible]

the heart muscle were added to the peripheral effects, and both factors undoubtedly contributed to the lethal ending. That relatively small increases in plasma potassium may predispose to vagal heart failure without causing the characteristic electrocardiographic disturbances of potassium poisoning is suggested by a recent report by Hoff, Humm, and Winkler (16).

It is difficult to say whether cardiovascular failure or respiratory insufficiency was the immediate cause of death. Profound arterial hypotension and pronounced bradypnea were usually encountered at the same time. It can be said, however, that the mean arterial pressure fell considerably before any impairment in respiratory function was evident. Artificial respiration applied at a time when the arterial pressure was still appreciable had no effect upon its downward course. Moreover, curarized pigs did not survive longer than spontaneously breathing animals; all but one animal died after 25 to 51 minutes of continuous immersion. The plasma potassium level increased by 66 to 250 per cent; the highest level found was 10.2 meq. per L. No profound changes in cardiac function, as judged by the electrocardiogram, occurred. As will be shown, plasma potassium levels up to 10 meq. per L. do not produce significant changes in intraventricular conduction.

At immersion temperatures of 60° to 75° C., the pigs survived for only 8 to 15 minutes. In the middle, or even earlier during exposure, at a time when the respiration was still adequate and the mean arterial pressure was still considerable, pronounced changes in cardiovascular function made their appearance. They consisted of a rise in right auricular pressure, and electrocardiographic changes in the form of disappearance of the P wave and progressive widening of the QRS complex, often terminating in ventricular fibrillation. At the same time, the potassium concentration of the plasma reached values of 16 to 19 meq. per L. This was associated with a striking destruction of red blood cells.

These observations strongly suggest that the hyperpotassemia was responsible for the disturbances in cardiac mechanism and for the subsequent myocardial failure, evidenced by the rise in auricular pressure. That the damaging effects of a rising plasma potassium level first of all manifest themselves in the heart is well known. In rabbits and dogs, the infusion of a solution of a potassium salt produces a sequence of electrocardiographic changes similar to those observed in pigs during exposure to high temperatures [Winkler, Hoff and Smith (17), and Nahum and Hoff (18)]. It was found that an identical sequence of changes takes place in infused pigs (Table IV). In 2 animals, infusion rates were maintained that were likely to produce death in approximately the same time as in the burned pigs. It is evident that potassium levels of less than 10 meq. per L. failed to produce either changes in the P wave or widening of the QRS complex, just as was the case in burned pigs. Higher levels resulted in a succession of changes which were similar in all respects to those observed at high temperatures (Table II). In the one animal (Figure 5) in which arterial and right auricular pressure and respirations were recorded, the auricular pressure began to rise 19 minutes (s) after the infusion had started. The potassium level was 12.7 meq. per L.; the P waves had begun to flatten 3 minutes before and had disappeared. Three minutes later widening of the QRS complex began. The arterial pressure and respiration remained normal for another 10 minutes.[3]

That the cardiac changes due to the potassium ion are reversible to a remarkable degree is clear from experiment 901 (Table IV). The usual succession of electrocardiographic changes was observed until, some seconds after a potassium level of 15.5 meq. per L. had been reached, the string shadow remained resting. The infusion was stopped. No electric or auscultatory evidence of cardiac activity could be demonstrated for the following 10 minutes, although the animal continued to breathe at a very slow rate. Then heart action returned and respiration became more rapid. The electrocardiogram had returned to normal. A plasma sample taken 5 minutes thereafter contained 8.7 meq. per L. of potassium. Infusion was started again, the well-known changes were again observed, and the pig died with a potassium level of 17.7 meq. per L.

The rapidity with which potassium is removed from the plasma makes it imperative that the re-

[3] The rate of infusion was slow enough so that the rise in venous pressure could not be ascribed to the administration of the isotonic salt solution *per se* [Altschule and Gilligan (19)].

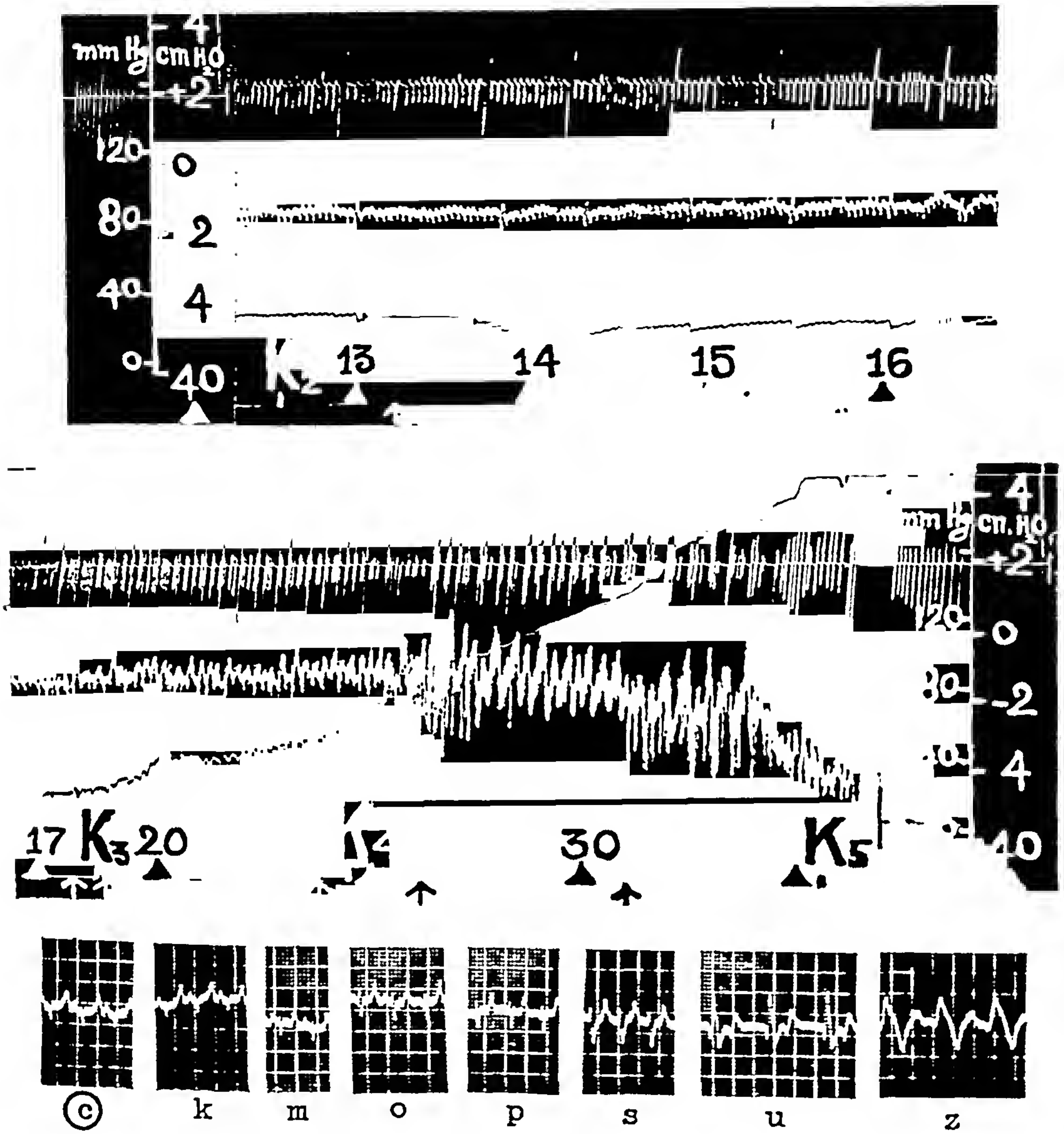

FIG. 5. EFFECT OF CONTINUOUS INTRAVENOUS INFUSION OF 1.12 PER CENT KCl AT THE RATE OF 0.6 ML. PER KGM. PER MIN.

The upper, middle, and lower tracings on the kymograph record represent respectively the pneumogram, the carotid pressure, and the right auricular pressure. The time in minutes is shown at the base of the record. The time at which blood samples were taken is indicated by the symbols K_2, K_3, K_4, and K_5. The times at which the depicted electrocardiograms were taken are indicated by letters from k to z.

lease of the ion into the circulation be intensive enough and be continued for a sufficiently long time to lead to death. This actually occurs in the burned pigs. The liberation of potassium often occurred at so rapid a rate that there was a lag between the rise in potassium and the electric changes. Thus, in pig 910, a level of 10.0 meq. per L. was reached in 2 minutes, whereas more than 4 minutes were required to produce the typical widening. Animals exposed to high temperatures for only 1 or 3 minutes did not release sufficient potassium to produce a characteristic effect on the heart; whereas exposure for 6.5 minutes was adequate in this respect. Exposure to 75° C. for 5 minutes resulted in a tremendous rise in potassium and in electrocardiographic changes, for

even here both manifestations diminished in intensity during the following 14 minutes.

Although it is clear that in pigs exposed to high (60° to 75° C.) temperatures the most striking physiological disturbances are those which result from the release of excessive amounts of potassium, continued exposure results in a progressive and generalized rise in body temperature which undoubtedly causes disturbances other than those due to hyperpotassemia. Thus, the peripheral and central factors that were the cause of death at lower temperatures also come into play at these high temperatures.

In order to evaluate the relative contributions of red blood cells and fixed body cells to the increase in plasma, potassium experiments were performed on dogs (Table III). Whereas the potassium concentration of their fixed cells is similar to that of the pig, their red cells contain only small amounts. Immersion at 75° C. resulted in an intense hemolysis, but the potassium level did not rise above that encountered in pigs at 47° C., and electrocardiographic changes characteristic of hyperpotassemia were not seen.

The distribution of the potassium in human blood is similar to that in pig's blood, the potassium concentration of the red cells being approximately 110 meq. per L., that of the plasma approximately 4 to 5 meq. per L. [Kramer (20), Scudder (21), and others]. High plasma potassium levels should therefore be expected in humans in whom a major part of the body surface has been exposed to high environmental temperatures. Several minutes of exposure would probably be required to result in the very high levels encountered in these experiments. It is also probable that, if the immediate effects of the exposure were survived, a markedly elevated plasma potassium, occurring immediately following the injury, would fall within the next hour. It should be remembered, of course, that a rise in plasma potassium is a normal postmortem phenomenon.

SUMMARY

There are 2 principal mechanisms by which exposure of the surface of the body to excessive heat may cause rapid circulatory failure and death.

In one, the systemic hyperthermia caused by conduction of heat to the interior of the body by way of the blood stream leads to a rapid and progressive decline in blood pressure and failure of circulation due principally to peripheral vascular collapse.

In the other, the circulatory failure is principally central and is due to the effect on the heart of an excessively high concentration of potassium in the plasma. Central circulatory failure is likely to occur when the overheating of the skin and subcutaneous tissue is so intense, prolonged, and generalized that potassium is released from the erythrocytes so rapidly and in such large amounts as to result in maintained plasma levels in excess of 11 meq. per L.

In the case of thermal exposures of low intensity, peripheral circulatory failure may occur without sufficient rise in tissue (and blood) temperature to cause a dangerous rise in plasma potassium. When a thermal exposure has been of sufficient severity to cause fatal hyperpotassemia, the central circulatory effects are likely to be complicated by peripheral vascular collapse.

It is essential to the development of hyperthermic potassium poisoning that the erythrocytes have a high original concentration of this element. Thus, fatal hyperpotassemia, due to hyperthermia, may occur in the pig but not in the dog. Since man and pig have similar potassium concentrations in their erythrocytes, it is inferred that they are probably similarly susceptible to the development of fatal hyperpotassemia following cutaneous exposures to excessive heat.

Although thermally induced disturbances of the respiratory centers may contribute to either type of hyperthermal circulatory failure, maintenance of pulmonary ventilation by artificial respiration does not prevent death or cause significant prolongation of the survival period.

BIBLIOGRAPHY

1. Moritz, A. R., Henriques, F. C., Jr., Dutra, F. R., and Weisiger, J. R., Studies of thermal injury. IV. Exploration of casualty-producing attributes of conflagrations. The local and systemic effects of generalized cutaneous exposure to excessive circumambient (air) and circumradiant heat of varying duration and intensity. Arch. Path. In press.
2. McLean, R., Moritz, A. R., and Roos, A., Studies of thermal injury. VI. Hyperpotassemia caused by cutaneous exposure to excessive heat. J. Clin. Invest., 1947, 26, 497.

3. Heymans, C., La tachycardie et la tachypnée pendant l'hyperthermie par le bleu de méthylène. Arch. Internat. d. Pharmacodyn. et Therap., 1923, 28, 51.
4. Uyeno, K., Studies on the respiration and circulation in the cat. III. The effects of rise of body temperature. J. Physiol., 1923, 57, 203.
5. Cheer, S. N., The effects of high temperature on the heart and circulation in intact animals. Am. J. Physiol, 1928, 84, 587.
6. Wiggers, C. J., and Orias, O., The circulatory changes during hyperthermia produced by short radio waves (radiothermia). Am. J. Physiol., 1932, 100, 614.
7. Ferris, E. B., Jr., Blankenhorn, M. A., Robinson, H. W., and Cullen, G. E., Heat stroke: clinical and chemical observations on 44 cases. J. Clin. Invest., 1938, 17, 249.
8. Fick, A., Hat Veränderung der Temperatur des im Hirn Cirkilirenden Blutes Einfluss auf die Centra der Herz- und Gefässnerven? Pflüger's Arch. f. d. ges. Phys., 1872, 5, 38.
9. Cyon, E., Ueber den Einfluss der Temperatur-änderungen auf die centralen Enden der Herznerven. Pflüger's Arch. f. d. ges. Phys., 1874, 8, 340.
10. Kahn, R. H., Ueber die Erwärmung des Carotidenblutes. Arch. f. Anat. u. Physiol. Leipzig Suppl. Bd. 81, 1904.
11. Moorhouse, V. H. K., Effect of increased temperature of the carotid blood. Am. J. Physiol., 1911, 28, 223.
12. Heymans, C., and Ladon, A., Recherches physiologiques et pharmacologiques sur la tête isolée et le centre vague du chien. I. Anemie, asphyxie, hypertension, adrenaline, tonus pneumogastrique, hyperthermie. Arch. Internat. de Pharmacodyn. et Therap., 1925, 30, 415.
13. Knowlton, F. P., and Starling, E. H., The influence of variations in temperature and blood pressure on the performance of the isolated mammalian heart. J. Physiol., 1912, 44, 206.
14. Shen, S. C., Ham, T. H., and Fleming, E. M., Studies on destruction of red blood cells. III. Mechanism and complications of hemoglobinuria in patients with thermal burns: Spherocytosis and increased osmotic fragility of red blood cells. New Eng. J. Med., 1943, 229, 701.
15. Harris, A. S., and Randall, W. C., Mechanisms underlying electrocardiographic changes observed in anoxia. Am. J. Physiol., 1944, 142, 452.
16. Hoff, H. E., Humm, D. G., and Winkler, A. W., Concentration of potassium in serum and response to vagal stimulation in the dog. Am. J. Physiol., 1944, 142, 627.
17. Winkler, A. W., Hoff, H. E., and Smith, P. K., Electrocardiographic changes and concentration of potassium in serum following intravenous injection of potassium chloride. Am. J. Physiol., 1938, 124, 478.
18. Nahum, L. H., and Hoff, H. E., Observations on potassium fibrillation. J. Pharmacol. and Exper. Therap., 1939, 65, 322.
19. Altschule, M. D., and Gilligan, D. R., The effects on the cardiovascular system of fluids administered intravenously in man. II. The dynamics of the circulation. J. Clin. Invest., 1938, 17, 401.
20. Kramer, B., and Tisdall, F. F., Distribution of sodium, potassium, calcium and magnesium between corpuscles and serum of human blood. J. Biol. Chem., 1922, 53, 241.
21. Scudder, J., Smith, M. E., and Drew, C. R., Plasma potassium content of cardiac blood at death. Am. J. Physiol., 1939, 126, 337.

STUDIES OF BREATHING, PULMONARY VENTILATION AND SUBJECTIVE AWARENESS OF SHORTNESS OF BREATH (DYSPNEA) IN NEUROCIRCULATORY ASTHENIA, EFFORT SYNDROME, ANXIETY NEUROSIS[1]

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Patients with neurocirculatory asthenia (N.C.A.), anxiety neurosis, or effort syndrome commonly complain of shortness of breath, inability to draw a satisfactory breath, and incapacity for work caused by breathlessness. Such symptoms have been noted in the classic works on this subject by DaCosta (1) and Sir Thomas Lewis (2) among others.

The present report deals with studies carried out to investigate in a quantitative way characteristics of breathing in N.C.A. These studies included investigations of (*1*) patients' symptoms as compared with those of control subjects; (*2*) study of characteristics of breathing at rest, including respiratory rate, minute respiratory volume, tidal air, oxygen consumption, incidence of sighs, variation in respiratory rate and in respiratory depth; (*3*) studies of breathing during exercise, of ventilation index, and of the relation of ventilation index to awareness of shortness of breath, *i.e.*, dyspnea; (*4*) studies of ventilation and ventilatory efficiency while walking and running on a treadmill. Details of technique will be described in each section.

In general, studies were done on 74 patients with chronic N.C.A. and on 27 patients with acute N.C.A. These two groups of patients were differentiated on grounds of history alone (3). Those who had a lifelong course and could never do hard work or athletics were designated as chronic N.C.A. Those who gave convincing evidence of good health, ability to do muscular work or athletics, and emotional stability previous to the development of illness were designated as acute N.C.A. In addition to studies related to breathing the patients were subjected to a wide variety of other tests which are reported elsewhere (3).

For control subjects we used 129 healthy subjects, of whom 117 were soldiers, and 25 soldiers who were convalescing from infected war wounds.

Incidence of symptoms

Method. A series of questions was administered to 74 patients with chronic N.C.A., 25 patients with acute N.C.A., 25 convalescent controls, and 55 healthy control subjects. The presence of the symptom was noted on a printed check list by plus, the absence by zero.

In addition, during the 20-inch step test (4, 5), patients and control subjects were asked to perform for 5 minutes at the pace of 30 trips per minute for a maximum of 5 minutes. The subjects were asked what stopped them when they did not go on for the full 5 minutes. The incidence of breathlessness among other symptoms was tabulated for control subjects and patients.

Results. The incidence in N.C.A. of symptoms involving breathing is presented in Table I, which shows that there is a high incidence of symptoms related to breathing in N.C.A. It is of further note that there is consistently, for each symptom, a higher incidence in the chronic N.C.A. group than in the acute; there is a slightly greater incidence of such symptoms as breathlessness and inability to get a satisfactory breath in convalescent subjects as compared to healthy controls.

The feeling of breathlessness may be present at any time in the patients. In addition, patients commonly have the feeling that they do not breathe in a satisfactory manner, that they do not get in enough air while breathing, or that their breathing does them no good.

Difficulties in breathing are quite marked when the patients attempt to wear a gas mask or while they are attempting to swim.

[1] This work was done under contract recommended by the Committee of Medical Research of the Office of Scientific Research and Development and the Massachusetts General Hospital.

With the technical assistance of Mary P. Lennon, Audrey Y. Dennison, and Jane R. Brown.

TABLE I

Incidence in neurocirculatory asthenia of symptoms involving breathing *

Group studied	Patients with N.C.A.		Controls	
	Chronic	Acute	Conv.	Healthy
Number of subjects	74	25	25	55
From present illness				
1. Breathlessness	99	79	24	13
2. Inability to get a satisfactory breath	74	41	16	2
3. Panting	62	52	4	5
4. Sighing	61	39	12	9
5. Gas mask trouble	55	17	12	15
6. Smothering	50	28	8	5
From past history				
7. Trouble breathing in water (while swimming)	75	22	12	10
8. Always short of breath	41	0	8	7
During hard work test				
9. Breathlessness severe enough to cause patients to stop	62		18	

* Significance of differences in incidence of symptoms: Chronic N.C.A. vs. healthy controls—all differences significant; chronic N.C.A. vs. acute N.C.A.—all differences significant except for 3, 4 and 6; chronic N.C.A. vs. convalescent controls—all differences significant; acute N.C.A. vs. convalescent control subjects—all differences significant except 2, 5, 6 and 7; acute N.C.A. vs. healthy control subjects—all differences significant except for 5 and 7; convalescent control subjects vs. healthy control subjects—only 2 show significant difference.

When patients and subjects were questioned as to what stopped them in the 20-inch step test, 60 per cent of the patients reported that they were winded or that their wind gave out, in contrast to 18 per cent of the healthy controls. This was true even though the healthy controls lasted much longer in the performance of the test.

Summary. N.C.A. patients have many complaints referable to breathing; symptoms are of higher incidence in chronic N.C.A. When questioned at the end of hard exercise, many N.C.A. patients state that shortness of breath causes them to stop before the test is finished.

Studies of characteristics of breathing at rest

Method. Respiratory measurements were made after 30 minutes rest, in a fasting condition and while breathing oxygen, in 50 men with N.C.A. and in 34 healthy men. The usual procedure for doing a basal metabolic rate test was employed (6) using a 12-minute record. The measurements were as follows: (*1*) Minute respiratory volume in liters of oxygen breathed per minute—this was obtained by measuring each inspirational length in a minute, multiplying the sum by the appropriate calibration factor. (*2*) Minute respiratory volume was adjusted for body surface by dividing by surface area obtained from height and weight tables (7). (*3*) Respiratory rate was counted in breaths per minute. (*4*) Tidal air was calculated for each minute by dividing ventilation per minute by respiratory rate per minute. The mean tidal air for the record was obtained from averaging the tidal air for each minute. (*5*) Tidal air was also adjusted for body surface by dividing the mean tidal air for each patient by his surface area. (*6*) A sigh was defined for purposes of this study as an inspiration which was at least 2½ times the depth of the previous inspiration if it was representative; if not, the first such breath in that minute was used as a basis of comparison. (*7*) Basal metabolic rate was calculated in the usual manner (6). (*8*) Coefficient of variation was calculated for rate in each patient by dividing the standard deviation of respiratory rate by the mean respiratory rate. Table II presents the mean of the coefficient of variation for respiratory rate. (*9*) Coefficient of variation for depth of inspiration was obtained by dividing standard deviation of the depths of inspiration for 6 minutes by the mean inspiratory depth (tidal air). The latter two measurements were made to discover whether the breathing of patients with N.C.A. was more irregular in rate and in inspiratory depth than was true of healthy control subjects.

Results. Table II shows that minute respiratory volume and oxygen consumption are the same for the two groups. However, respiratory rate is definitely more rapid in patients, and the breathing in patients is more shallow. This is true even after adjusting for differences in body size. Sighing is more frequent in patients than in control subjects, but not significantly so in this series.

Summary. N.C.A. patients resting, fasting, and breathing oxygen as compared with healthy control subjects show: (*1*) a higher respiratory rate, (*2*) shallow breathing, (*3*) a greater coefficient of variation in depth of inspiration.

TABLE II
Breathing measurements

	Units	Healthy controls	N.C.A. patients	Signif. ratio
Number of subjects		34	50	
Minute respiratory volume	*Liters per minute*	8.52	8.32	
Minute respiratory volume	*Liters per minute and meter*^{2}	4.77	4.53	1.42
Respiratory rate	*per minute*	13.2	15.8	2.00
Tidal air	*ml.*	743	553	
Tidal air per m^2 body surface	*ml. per meter*^{2}	386	306	3.48
Sighs	*per minute*	.08	.15	.02
Basal metabolic rate	*per cent*	−1	−4	.09
Coefficients of variation				
Mean of respiratory rate		8.34	8.28	3.69
Mean of depth of respiration		20.25	25.12	1.71

the feeling of shortness of breath correlate with measurable changes in ventilation or vital capacity, the usual correlates of dyspnea (8, 9)? (*2*) Do patients with N.C.A. show differences in minute respiratory volume during and after comparable amounts of varying degrees of exercise as compared with healthy control subjects? (*3*) Is ventilation index, an objective measure proposed by Harrison and associates (10) as a quantitative objective correlate of dyspnea, abnormal in N.C.A.? (*4*) Do N.C.A. patients develop dyspnea at an abnormal level of ventilation index?

Method. Ventilation index studies described by Harrison and associates (10) were done in 58 patients with N.C.A. and in 55 healthy soldiers. Formula for ventilation index is:

$$\text{Ventilation index} = \frac{\text{ventilation}}{\text{vital capacity}} \times \frac{1 + \dfrac{\text{ideal weight}}{\text{actual weight}}}{2}.$$

All control subjects were healthy American soldiers. The N.C.A. patients consisted of 33 soldiers, 16 sailors, 2 marines, and 7 civilians. There were 41 cases of chronic N.C.A. and 17 of acute N.C.A.

The test was carried out by having the subject walk over a set of steps with measurements of ventilation before, during, and after the exercise. The steps used have been described by Harrison and have the following measurements: low step 17 cm., high step 32 cm., length of platform 64 cm.

The patient was given a demonstration of the 4 paces of the test. He practiced a few steps at each pace in order to familiarize himself with the steps. He then sat in an Adirondack reclining chair for 30 minutes. At the end of this period the patient was connected to the Tissot spirometer. The resting ventilation for 3 minutes was measured along with pulse rate and respiratory rate. The exercise then proceeded for 2 minutes and the patient then rested for a 5-minute recovery period, the ventilation being measured continuously during this 7-minute period (comprising 2 minutes exercise and 5 minutes recovery period). The pace of the step test was regulated by a metronome. The first exercise consisted of 6 trips, the second of 12 trips, the third of 16 trips, and the fourth of 24 trips in 2 minutes. One observer, using a stopwatch, counted off the pace for the patient, slowed him down or speeded him up if he lost the pace, held the tubing connecting him to the Tissot to avoid his being entangled in it, and for certainty checked the number of trips with a Veeder counter. Second observer read and recorded the Tissot units and also counted respiratory rate during the test.

Pulse recovery rate was determined also by the first observer. The pulse was counted after the patient sat down from 1 to 1½ minutes, 2 to 2½ minutes, and 4 to 4½ minutes. At the end of each 5-minute recovery period the patient was confronted with a printed card with the questions: Were you short of breath? Not at all? A little? Moderately? Severely? Were you very uncomfortable or did you feel distress? Yes or No. The patient was asked to point out the answer to each question which described best his subjective feelings during the test. In addition to this, one of the observers tried to estimate whether the patient seemed to be in respiratory distress—slightly, moderately, severely, or not at all. During the 1 minute between tests when the patient reported his subjective feelings by pointing to the question card, the Tissot level was readjusted, if necessary; the metronome was set to the next speed; and the patient was asked to stand about 15 seconds before the beginning of the next test. Then the next exercise began.

Vital capacity was determined on the patient in a sitting position. This was carried out by the method previously described (11), the highest of 3 determinations checking within 50 ml. being accepted. Age, height, and weight of the patient were determined and surface area was taken from standard Benedict-Roth tables (7). Room temperature, Tissot temperature, and barometric pressure were recorded.

Results. *Ventilation index:* Figure 1 shows that for each exercise the mean Ventilation Index is higher in the N.C.A. patients than it is in the healthy control subjects. The differences between

VENTILATION INDEX

	NORMAL 54	PATIENT 56
I SLOW	15.4	18.7
II MILD	18.3	23.2
III BRISK	20.7	26.2
IV FAST	28.6	36.9

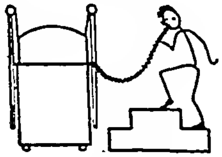

Fig. 1

The means of ventilation index for 56 N.C.A. patients as compared with 54 control subjects are compared above, for the 4 paces of exercise. The ventilation index is significantly higher in N.C.A. for each exercise. The differences were progressively greater as the speed of exercise increased. The figure illustrates schematically the patient during exercise breathing to the Tissot spirometer.

the controls and the patients are statistically significant (see Tables III and IV).

It is of further interest that patients with chronic N.C.A. show higher ventilation indices than do patients with acute N.C.A., this difference being consistent for all 4 exercises.

TABLE III

Ventilation index—means and statistics in neurocirculatory asthenia and healthy controls

Exercise	No	Controls V.I.	Controls 6	S.E.	No.	N.C.A. V.I.	N.C.A. 6	S.E.	S.E.diff.	Signif. ratio	Odds
I	53	15.4	2.39	.328	56	18.7	6.67	.892	.950	3.53	2,200–1
II	54	18.3	2.39	.325	56	23.2	9.09	1.216	1.257	3.86	8,000–1
III	54	20.7	2.81	.353	56	26.2	10.35	1.383	1.435	3.85	8,000–1
IV	54	28.6	4.11	.559	56	36.9	13.15	1.758	1.849	4.50	20,000–1

No. = number of subjects exercised; V.I. = Ventilation Index; 6 = Standard deviation of the observations in the sample; S.E. = the standard error of the mean; $S.E._{diff.}$ = standard error of the difference between the two means; Significance ratio = $S.E._{diff.}$ ÷ difference between means; Odds = the probability of the difference arising once in this number of times by chance.

TABLE IV

Ventilation index

Acute N.C.A. (17 cases) compared with chronic N.C.A. (39 cases)

	Acute N.C.A.	Chronic N.C.A.
Exercise I	16.9	19.6
Exercise II	21.4	24.0
Exercise III	23.9	27.3
Exercise IV	34.7	37.9

Vital capacity: The high ventilation index in N.C.A. is not due to abnormal vital capacity, as vital capacity in N.C.A. does not differ from vital capacity of healthy control subjects of similar size; Table V shows that the vital capacity in ml. and per square meter of body surface is 2,419 for healthy control subjects, 2,426 for N.C.A. patients.

TABLE V

Vital capacity

(Neurocirculatory asthenia vs. healthy controls)

	Number	V.C.	V.C.
		ml.	*ml. M²*
Controls	54	4,443.3	2,419.4
N.C.A.	54	4,395.1	2,425.5

Vital Capacity—Standard Error of Difference between means = .92; significance ratio = .52. Vital Capacity per meter squared of body surface—Standard Error of Difference between means = 50.9; significance ratio = .12. Thus, neither the slight differences in vital capacity nor those in vital capacity per M² between N.C.A. and controls are statistically significant.

The slight differences in vital capacity between N.C.A. and healthy controls are obviously not significant. The significance ratio of the differences for vital capacity is .52, for vital capacity per square meter is .12.

Although vital capacities were the same in value in the two groups, it was of interest that more trials were required to obtain 3 checking values in N.C.A. A mean of 3.3 extra trials was required to check the controls, as against 5.44 extra trials for patients. This difference was significant, significance ratio being 2.36. Two controls and 12 patients never checked within 50 ml.; in those we used the highest value for calculation purposes.

Total ventilation during and after exercise: The total of ventilation (2 minutes during and 5 minutes of recovery after exercise) is shown in Table V to be higher for each exercise, in N.C.A. than in healthy control subjects. The mean ventilations in liters for 7 minutes are: *Exercise I*—Controls 72.3, N.C.A. 85.2; *Exercise II*—Controls 86.0, N.C.A. 105.8; *Exercise III*—Controls 97.2, N.C.A. 119.8; *Exercise IV*—Controls 13[illegible].2, N.C.A. 169.8. The differences between N.C.A. cases and controls become greater as the exercise pace increases.

The difference in ventilation index between N.C.A. and control subjects is thus due to the ventilation factor, not to the vital capacity factor.

Minute respiratory volume during and after exercise: Figure 2 shows that for each minute during and after exercise the mean minute respiratory volume is higher in N.C.A. than in healthy controls. The differences become greater as the pace of exercise becomes more rapid. Figures 2 and 3 further illustrate that the differences are more marked after exercise than during exercise. The percentage difference between the mean ventilation in N.C.A. and in healthy control subjects, using the mean ventilation of the healthy subjects for that minute as a baseline, reaches its peak during the second recovery minute, as shown in Figure 4.

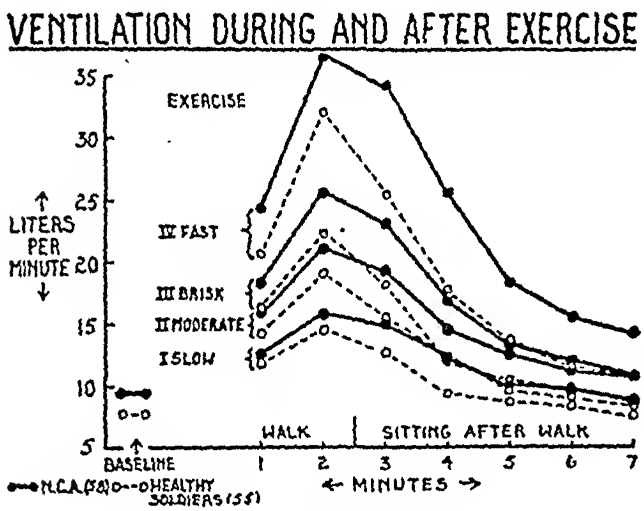

FIG. 2

The ordinate shows liters per minute of pulmonary ventilation; on the abscissa are marked off the time intervals during 2 minutes of exercise and 5 minutes of recovery. For all minutes and all exercises the patients show higher minute respiratory volume than do the controls.

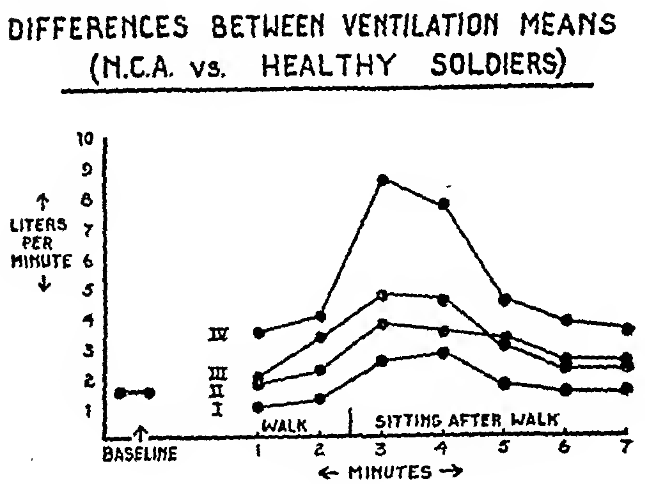

FIG. 3

The ordinate shows the excess between minute respiratory volume for each minute and each exercise in N.C.A. The abscissa marks off the difference at rest (baseline), and the time intervals during 2 minutes of exercise and 5 minutes of recovery. Roman numerals indicate progressively increasing speeds of exercise. The differences are more marked in the early recovery period.

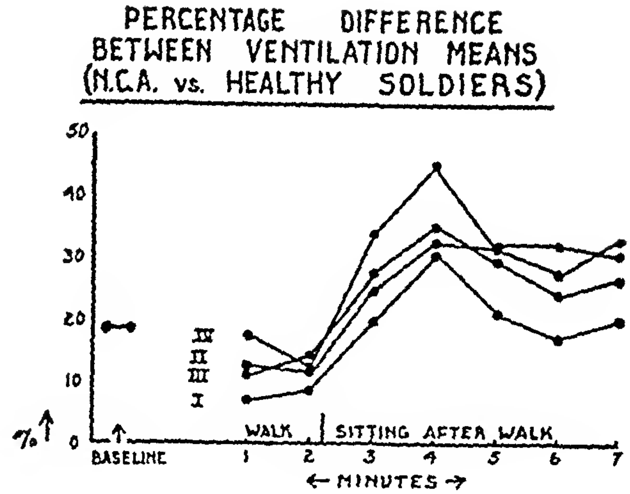

FIG. 4

The ordinate shows the percentage difference between ventilation means of N.C.A. and controls, using the controls as a basis of comparison, for each minute and each exercise. The abscissa marks off the time intervals, baseline indicating the initial resting value, and the time intervals during 2 minutes of exercise and 5 minutes of recovery. Roman numerals indicate progressively increasing speeds of exercise. The percentage differences are more marked in the early recovery period.

Respiratory rate and tidal air: The respiratory rate was greater in N.C.A. than in controls. Table VI shows that this is true during the preliminary baseline observations and for each exercise, the data for the fourth minute (*i.e.*, second recovery

TABLE VI

Respiratory rate and tidal air
During 4th minute of test, i.e., 2nd recovery minute

Exercise	Number		Respiratory rate		Tidal air	
	Controls	N.C.A.	Controls	N.C.A.	Controls	N.C.A.
			per minute		*ml. per M²*	
Baseline	53	49	15.3	17.2	293	317
I	31	55	15.4	18.4	343	377
II	28	54	16.5	20.4	386	420
III	26	52	16.2	21.3	423	460
IV	37	51	17.6	22.9	583	634

TABLE VII

Dyspnea, distress, and exercise in 55 N.C.A. patients and 54 control subjects

Degrees of dyspnea associated with each rate of exercise
(per cent incidence—degrees of dyspnea)

	None		Slight		Moderate		Severe	
	Controls	N.C.A.	Controls	N.C.A.	Controls	N.C.A.	Controls	N.C.A.
Exercise I	83	29	13	49	4	18	0	4
Exercise II	67	9	26	40	6	38	0	13
Exercise III	54	8	37	31	9	45	0	16
Exercise IV	26	4	33	14	33	24	7	58

Per cent incidence of dyspnea and of distress or discomfort associated with each rate of exercise

	Dyspnea			Distress		
	Controls	N.C.A.	Significance ratio	Controls	N.C.A.	Significance ratio
Exercise I	17	71	3.5	9	30	2.6
Exercise II	31	91	3.9	14	61	5.2
Exercise III	46	92	3.8	14	67	6.0
Exercise IV	74	96	4.5	25	73	5.1

minute) being presented. Table VI also shows that the tidal air, adjusted for body size, is greater in N.C.A. during the recovery period after each exercise.

Calculation reveals that the maximum percentage rise in respiratory rate is 33 per cent in N.C.A. as compared with 15 per cent in controls; the maximum rise in tidal air is 100 per cent in N.C.A. as compared with 99 per cent in controls. This shows that the differences in minute respiratory volume between N.C.A. and controls are largely due to the disproportionate rise in respiratory rate in N.C.A. The maximum difference in tidal air during this time interval between N.C.A. and controls is 10 per cent, in respiratory rate 32 per cent.

Subjects' awareness of dyspnea and distress as related to level of ventilation index (see Table VII): Table VIII shows that for the same range

TABLE VIII

Percentage incidence of "dyspnea" and "distress" at ventilation indices 15 through 25

	Shortness of breath				Distress
	None	Slight	Moderate	Severe	
Controls	63	28	8	1	15
N.C.A.	13	49	32	68	51

of ventilation index a higher incidence of patients reports shortness of breath and distress or discomfort as compared with controls. Not only do more patients feel shortness of breath but they also report feeling a greater degree of shortness of breath. For example, at this level of ventilation index 68 per cent of N.C.A. patients felt severe shortness of breath in contrast to only 1 per cent of healthy controls.

This demonstrates that at the same level of ventilation index, the "objective correlate of dyspnea," more patients feel distress and shortness of breath, and to a greater degree, than do control subjects. This shows that the incidence and degree of dyspnea in N.C.A. is not only out of proportion to the amount of exercise, but also is out of proportion to the amount of ventilation and ventilation index.

Appearance of objective respiratory distress: Table IX shows that by our impressionistic method of estimating the presence and degree of objective respiratory distress N.C.A. patients showed a higher incidence of objective respiratory distress than controls for the same amount of exercise. At the same amount of ventilation index, the patients exhibited a higher incidence of appearance of objective respiratory distress with a greater degree of it.

TABLE IX

Appearance of respiratory objective distress in 54 N.C.A. patients and 54 healthy controls

Percentage incidence of appearance of objective distress during each exercise

Exercise	Controls	N.C.A.
I	11	58
II	33	91
III	50	96
IV	89	100

Percentage incidence of appearance of objective distress and its severity for ventilation indices 15 through 25

	Percentage incidence of appearance of objective respiratory distress			
	None	Slight	Moderate	Severe
Controls	60	29	11	0
N.C.A.	19	58	21	9

When we compared our estimates of the 3 degrees of severity of objective breathlessness with the 3 degrees of the patients' subjective reports, there was noted a tendency for the patients to report feeling more than we estimated, for the controls to report less. This comparison is obviously impressionistic, and this must be taken into consideration when evaluating this type of data.

SUMMARY. 1. Ventilation index is higher in patients for each exercise than in controls, the difference becoming greater as the rate and amount of exercise increase.

2. Vital capacity does not differ from that of controls.

3. Total pulmonary ventilation is higher, during and after exercise, the difference being greater as the rate and amount of exercise increase.

4. The high ventilation index in N.C.A. is due to the high ventilation, therefore, and not to the vital capacity factor.

5. The resting minute respiratory volume, with persons seated and breathing air, is slightly but significantly higher in patients.

6. Minute respiratory volume is higher in N.C.A. for each minute and for each exercise. The greatest minute respiratory volumes are noted at the beginning of the recovery periods, not during the exercise.

7. Both respiratory rate and tidal air are higher in N.C.A. than in controls, as judged from the second recovery minute.

8. The differences in minute respiratory volume between N.C.A. and controls are largely due to the disproportionate rise in respiratory rate in N.C.A.

9. For the same amount and rate of exercise patients complain in higher incidence and of a greater degree of shortness of breath than do controls.

10. For the same amount and rate of exercise patients complain in higher incidence of distress and discomfort.

11. For the same amount and rate of exercise more patients exhibited objective respiratory distress than did controls.

12. For the same level of ventilation index more patients exhibited objective respiratory distress than did controls.

13. In comparing our estimates of patients' objective respiratory distress with patients' subjective reports, there was noted a tendency for the patients to report more than we estimated, for the controls to report less.

14. Both the awareness of and outward appearance of shortness of breath are related in N.C.A. to some factor or factors other than amount of pulmonary ventilation.

Breathing while walking and running on a treadmill

METHOD. Ventilation and ventilatory efficiency were studied in N.C.A. patients while running at 7.0 miles per hour and while walking at 3.5 miles per hour on a treadmill, Grade 8.9 per cent [2] by methods described by Robinson (12). Other aspects of this study have been reported elsewhere (3, 13).

RESULTS. Table X shows that during running ventilation starts higher but ends lower; as the run progresses ventilatory efficiency is lower at all intervals of run for N.C.A. Data from a group of younger men (14) are included for comparison and the N.C.A. patients differ even more from them.

During the walk there is a higher pulmonary ventilation and a lower ventilatory efficiency in N.C.A.

SUMMARY. Ventilation is higher in N.C.A. for moderate work than in controls, is higher in N.C.A. as hard work begins but becomes lower as hard work progresses. Ventilatory efficiency is

[2] Referred to in previous Harvard Fatigue Laboratory publications as 8.6 per cent.

TABLE X

Pulmonary ventilation and ventilatory efficiency N.C.A. vs. controls

While running on a treadmill—7.0 miles per hour at 8.9 per cent grade, pulmonary ventilation in ml. per kilo and min.

	Age	No.	½–1 min.	1–2 min.	2–3 min.
Healthy men	20	46	730	1,027	1,231
Healthy men	29	20	645	977	1,216
N.C.A.	28	20	829	1,037	1,108

Ventilatory efficiency $\left(\frac{O_2 \text{ consumed}}{\text{Ventilation}}\right)$

	Age	No.	½–1 min.	1–2 min.	2–3 min.
Healthy men	20	46	5.34	4.90	4.23
Healthy men	29	20	5.29	4.59	3.92
N.C.A.	27	18	5.29	3.68	3.46

While walking on a treadmill, 3.5 miles per hour, 8.9 per cent grade

	No.	Ventilation	Ventilatory efficiency
		ml. per kilo and min.	
Healthy men	20	574	5.1
N.C.A.	20	657	4.5

lower in N.C.A. than in healthy controls for moderate and hard muscular work.

DISCUSSION AND SPECULATION

In neurocirculatory asthenia, anxiety neurosis, or effort syndrome many respiratory symptoms occur in high incidence. They constitute a characteristic and therefore diagnostic feature of the disorder; the absence of such symptoms makes the diagnosis of N.C.A. improbable. The complete mechanism of these symptoms is unknown. It is of interest that when respiration is investigated objective abnormalities are found, just as when other symptoms of N.C.A. are investigated with objective methods (3), which demonstrates that the abnormalities are not all in the subjective sphere.

The abnormalities at rest and breathing oxygen are few; while breathing air at rest M.R.V. was higher; during exercise the abnormalities become more pronounced, the deviations from the normal becoming greater as rate and amount of excercise increase. This is of interest particularly since the patients' disability involves especially hard work.

Ventilation index was higher in N.C.A. than in controls. This was true, but to a lesser degree, in cases of "cardiac neurosis" described by Harrison (15) who felt that the differences were not noteworthy. The differences between N.C.A. and controls are more marked in this study, probably due to the fact, as suggested to us by Harrison (16), that his healthy controls were less fit than ours and our N.C.A. patients were sicker clinically than those he studied. In fact, this corresponds with a general scheme in which the most fit and youngest subjects show lowest values, the healthy men in the late twenties are next, N.C.A. higher, the most severe being highest (Table XI).[10, 15, 17] Patients with heart disease, of course, are highest (10).

TABLE XI

Levels in ventilation index in various groups of healthy men and in N.C.A. patients

Subjects	No.	Exercise	
		II	IV
Athletes (Harrison, 17)	13	16.4	23.7
Medical students (Harrison, 17)	41	17.3	28.6
Healthy soldiers	54	18.4	29.0
Under 30 years of age (Harrison, 10)	10	20.8	34.8
Cardiac neurosis (Harrison, 15)	16	22.2	
Neurocirculatory asthenia	55	23.2	36.9

The ventilation factor accounts for the high ventilation index. The ventilation is highest after exercise, disproportionately so in N.C.A. as compared with controls. This may be related to other evidence of increased oxygen debt in N.C.A. and high blood lactate after exercise (3, 5, 13). It is possible that the higher blood lactate concentration during and after exercise in N.C.A. may participate as a stimulus to augmented ventilation. No crucial data are at hand that bear on these points. It is not known to what extent factors of physical training and patients' attitudes affect these results.

It is of further interest that the greater the stress or stimulus the greater and the more frequent the respiratory abnormality in N.C.A. This corresponds with patients' clinical history, with observations such as earlier ones made by one of us (White, 18) that running while wearing a gas mask was especially difficult and extremely abnormal in patients with effort syndrome and neurosis. The general rule that the greater the stimulus the more obvious the abnormality in N.C.A. is true of phenomena other than respiratory ones, for example, response to pain (3).

There has been a certain amount of discussion as to the proper definition of dyspnea. Means (19) defines it as a "symptom that arises whenever in carrying on the respiratory function difficulty is encountered." He points out that "when, however, the respiratory organs do meet with embarrassment in the performance of their task, then not only do their movements enter the field of consciousness, but they enter it unpleasantly and produce discomfort, that is to say dyspnea." Meakins (20) states "dyspnea is the consciousness of the necessity for increased respiratory effort." Harrison (10) regards dyspnea as a subjective phenomenon purely. Wright (21) states that "when the breathing enters consciousness unpleasantly and produces discomfort it is called dyspnoea." Christie (22) takes exception to this point of view, feeling that dyspnea should describe "difficult, painful, bad, or disordered breathing. The word can therefore be used objectively as well as subjectively and the more elastic definition of dyspnea as breathing associated with effort is fully justified." He points out that if one adopts too rigidly the definition of dyspnea as a subjective complaint "the absurdity is admitted that the hysterical patient who shows no evidence of increased ventilation or respiratory embarrassment but complains of inability to get enough air into the lungs is truly dyspneic; while the patient with pneumonia who, though distressed, refuses to admit a feeling of breathlessness is not dyspneic."

It is not clear to us what the proper usage of the word dyspnea should be. If the word is used at all, however, it should be used uniformly to mean the same thing by all observers. It is clear from this study that the subjective feeling of shortness of breath and distress is not based entirely on the amount of ventilation, since N.C.A. patients feel more shortness of breath and distress for the same amount of ventilation than do controls. This is true in addition to the fact that for the same amount of work N.C.A. shows higher ventilation and ventilation index.

It is important, therefore, that the subjective complaints of shortness of breath, distress, and discomfort be considered as not identical with the phenomenon of increased respiratory volume. There is, it might be added, no definite evidence as yet as to what area or areas of the brain are associated with the localization of the subjective feelings of dyspnea.

The tendency of N.C.A. patients to react more to uncomfortable stimuli is present in other spheres of discomfort, such as thermal pain and uncomfortable hand gripping (3, 5), and is also evidenced in other functions such as pulse rate (3). These facts do not explain the phenomenon but make it important to realize that it is not an isolated one, since N.C.A. patients react more than do controls to other types of stimulus and with other systems.

We were not able to confirm the finding of low vital capacity in effort syndrome patients in whom similar studies of respiration were made (23). These observers reported vital capacity mean of 2,840 ml. in patients and 4,172 ml. in controls. It is possible that difficulty in checking vital capacity results in N.C.A. may account for this difference. We found that patients and controls had identical vital capacity values as determined by our strict technique, but patients took more trials for a check. Whether this was due to poor motivation, poor voluntary control of respiratory movements, different type or stage of illness studied, or to other factors is unknown.

The evidence of poor ventilatory efficiency corresponds interestingly, although it may not explain, another symptom which patients have which is that they "can't get in enough air" or that "air doesn't seem to do as much good as it should."

CONCLUSIONS

1. In neurocirculatory asthenia, effort syndrome, or anxiety neurosis there are many symptoms referable to respiration.

2. At rest, while breathing oxygen, patients show rapid respiratory rate and abnormally shallow breathing.

3. While breathing air, minute respiratory volume is significantly higher in N.C.A. at rest and during moderate exercise than in controls, becoming lower as hard exercise progresses.

4. The greatest disproportion in pulmonary ventilation between patients and controls occurs in the early minutes of the recovery period after exercise, suggesting a relation to the high blood lactate and oxygen debt mechanism.

5. Vital capacity is normal in neurocirculatory asthenia.

6. Ventilation index, an objective correlate of dyspnea, is high in neurocirculatory asthenia.

7. The incidence and degree of dyspnea are out of proportion to the severity of exercise, the pulmonary ventilation, or the ventilation index in N.C.A.

8. Ventilatory efficiency is low in neurocirculatory asthenia both in moderate and in severe muscular exercise.

BIBLIOGRAPHY

1. DaCosta, J. M., On irritable heart; a clinical study of a form of functional cardiac disorder and its consequences. Am. J. M. Sc., 1871, 61, 17.
2. Lewis, T., The Soldier's Heart and the Effort Syndrome. Shaw and Sons, Ltd., London, 1940.
3. Cohen, M. E., Johnson, R. E., Chapman, W. P., Badal, D. W., Cobb, S., and White, P. D., A study of neurocirculatory asthenia, anxiety neurosis, and effort syndrome. Final report to the Committee on Medical Research of the Office of Scientific Research and Development under Contract OEM-cmr 157, 1946.
4. Brouha, L., Graybiel, A., and Heath, C. W., The step test: simple method for measuring physical fitness for hard muscular work in adult man. Rev. Canad. de Biol., 1943, 2, 86.
5. Cohen, M. E., Johnson, R. E., Cobb, S., Chapman, W. P., and White, P. D., Studies of work and discomfort in patients with neurocirculatory asthenia. J. Clin. Invest., 1944, 23, 934.
6. Hawk, Philip B., and Bergeim, Olaf, Practical Physiological Chemistry. Eleventh Edition. The Blakiston Company, Philadelphia, 1937.
7. Roth Metabolimetric Chart. Warren E. Collins, Boston. From Dubois and Dubois, Arch. Int. Med., 1916, 17, 865.
8. Peabody, F. W., and Wentworth, J. A., IV. The vital capacity of the lungs and its relation to dyspnea. Arch. Int. Med., 1917, 20, 443.
9. Peabody, F. W., and Sturgis, C. C., The relation of dyspnea to the maximum minute-volume of pulmonary ventilation. Arch. Int. Med., 1922, 29, 236.
10. Harrison, T. R., Turley, F. C., Jones, E., and Calhoun, J. A., Congestive heart failure. X. The measurement of ventilation as a test of cardiac function. Arch. Int. Med., 1931, 48, 377.
11. Thomson, K. J., and Cohen, M. E., Studies on the circulation in pregnancy. II. Vital capacity observations in normal pregnant women. Surg. Gyn. and Obst., 1938, 66, 591.
12. Robinson, S., Experimental studies of physical fitness in relation to age. Arbeitsphysiol., 1938, 10, 251.
13. Cohen, M. E., Johnson, R. E., Consolazio, F., and White, P. D., Low oxygen consumption and low ventilatory efficiency during exhausting work in patients with neurocirculatory asthenia, effort syndrome, anxiety neurosis. J. Clin. Invest., 1946, 25, 920.
14. Heath, C. W., Unpublished data.
15. Harrison, T. R., Harris, S., Jr., and Calhoun, J. A., Studies in congestive heart failure. XVI. The clinical value of the ventilation test in the estimation of cardiac function. Am. Heart J., 1931, 7, 157.
16. Harrison, T. R., Personal communication.
17. Turley, F. C., and Harrison, T. R., Respiratory measurements as affected by smoking and by athletics. Am. J. M. Sc., 1932, 183, 702.
18. White, P. D., Observations on some tests of physical fitness. Am. J. M. Sc., 1920, 159, 866.
19. Means, J. H., Dyspnoea. Medicine, 1924, 3, 309.
20. Meakins, J. C., Dyspnea. J. A. M. A., 1934, 103, 1442.
21. Wright, S., Applied Physiology (6th Edition). Oxford, New York, 1937.
22. Christie, R. V., Dyspnoea: a review. Quart. J. Med., 1938, 7, 421.
23. Jones, M. S., and Scarisbrick, R., A comparison of the respiration in patients with effort syndrome and in normal subjects. War Med., 1942, 2, 901.

TRAUMATIC SHOCK. XIV. THE SUCCESSFUL TREATMENT OF HEMORRHAGIC SHOCK BY VIVI-PERFUSION OF THE LIVER IN DOGS IRREVERSIBLE TO TRANSFUSION [1]

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The development of irreversibility to transfusion in the dog in hemorrhagic shock can be prevented by vivi-perfusion of the liver with arterial blood from a donor animal (1). The peculiar relationship of the liver to hemorrhagic shock is emphasized by the fact that vivi-perfusion via other channels does not prevent the development of irreversibility to transfusion. If the loss of integrity of the liver parenchyma is the key to the problem of peripheral circulatory collapse in shock, it becomes important to determine to what extent the damage to liver cells, if allowed to occur, may be corrected by restoring normal oxygen and volume flow requirements to the damaged liver cells. This problem was attacked by allowing the dog to develop irreversibility to transfusion and then perfusing the liver by cross-circulation with a donor dog. Repair of liver cell injury should then be reflected in a reversal of the rapidly progressive deterioration of the peripheral circulation characteristic of advanced shock. This communication will present data showing that hemorrhagic shock which has not responded to transfusion can be successfully treated by cross-circulation of the liver via the splenic vein with a healthy donor dog and that cross-circulation via a systemic vein is not effective.

METHOD

Hemorrhagic shock was induced in non-anesthetized dogs who had been given a single intramuscular injection of morphine (2 mgm. per kgm.). Incisions were made under local anesthesia. Those dogs which were to be perfused via the splenic vein were prepared 1 to 3 weeks in advance by splenectomy and subcutaneous implantation of a large splenic vein. In a few instances this vein was found to be occluded. Such dogs together with other normal dogs were used for perfusion via a systemic vein. Shock was induced by bleeding from a femoral artery attached to an elevated reservoir so that the blood pressure was maintained at 30 mm. Hg until "irreversibility" was considered to have developed in accordance with standards previously described (1). All shed blood was then rapidly infused via a femoral vein and the post-transfusion course was observed until it was clear that the animal had again relapsed into shock. At this time cross-circulation with a normal donor dog of about the same size was started (Figure 1). Blood from the donor dog's femoral artery, which was connected by means of clean (alkali-treated), but not sterile, rubber and glass tubing to the splenic or femoral vein of the dog in shock, was returned to the donor's femoral vein from the femoral artery of the dog in shock. Flowmeters were interposed in the delivery and return circuits. These were constructed as previously described (1) except that the dimensions of the return flowmeter were altered to accommodate the flow from the recipient at a higher pressure than obtained in the previous series (1). Continuing failure of the shocked dog's circulation was manifest by progressive decline in the recipient's blood pressure and evidence of exsanguination of the donor into the recipient. Both animals were maintained above shock levels of blood pressure by adding heparinized blood taken from other normal dogs. Cross-circulation was continued until the recipient dog was obviously terminal or had recovered sufficiently to maintain a normal blood pressure without added blood. Cross-circulation was then terminated. All vessels were ligated, sulfanilamide powder was placed in the wounds, and the incisions were closed.

Lactic acid, glucose, amino acid nitrogen, and non-protein nitrogen were measured in specimens of arterial blood by the methods previously used (1). Blood urea nitrogen was determined by the method of Karr (2).

RESULTS

Data on 9 liver-perfused and 9 control dogs are given in Tables I and II. No evidence of a toxic substance passing from the dog in irreversible shock to the donor was noted. Drops in donor blood pressure were considered as evidence of "taking-up" [2] by the recipient dog. When the vol-

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] If the dog in hemorrhagic shock bleeds into an elevated reservoir, connected to a large artery, until the falling

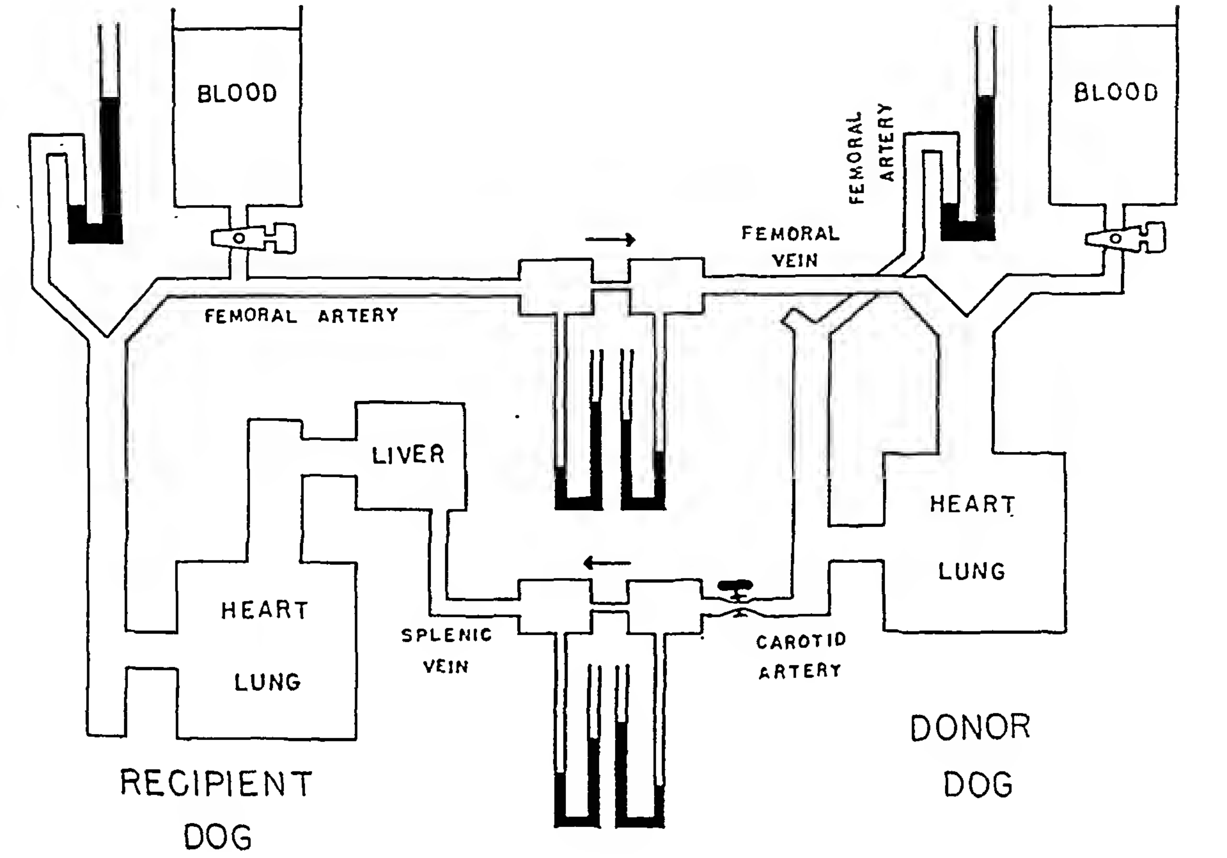

FIG. 1. DIAGRAMMATIC REPRESENTATION OF THE VASCULAR CIRCUIT IN LIVER PERFUSION

ume of blood lost to the recipient during the "taking-up" period was replaced and the donor's blood pressure returned to normal, the donor remained in good condition for the rest of the experiment. Several experiments in which technical accidents resulted in death of the donor dog were discarded.

blood pressure equals the hydrostatic pressure (30 mgm. Hg) in the reservoir, the dog remains stabilized at this pressure until after some time, varying from 1½ to 3 hours, when blood begins to return from the reservoir to the artery. The sustaining effect of this additional blood on the blood pressure is slight and short-lived and the "*taking-up*" of blood by the dog from the reservoir proceeds continuously or intermittently but with increasing speed until death. If one substitutes for the reservoir a donor dog in circuit with the dog in shock before the "taking-up" process begins or while it is in progress, the donor dog will serve as a reservoir and his blood pressure will fall as he exsanguinates himself into the recipient. The added blood pools in the recipient's peripheral vascular bed, which, having lost tone, dilates passively to receive the blood. This "taking-up" phenomenon, previously described in Paper XIII of the series (1), is an objective method of visualizing the progressive collapse of the peripheral circulation in the dog in shock.

The control dogs (Table I) showed little or no benefit from the cross-circulation. "Taking-up," which is an objective demonstration of paralysis of peripheral vascular tone and trapping of blood (3), not only continued after cross-circulation was started but often increased in the next hour or two so that it became difficult to sustain the blood pressure of the recipient (Table III). The blood pressure continued to fall in spite of the infusion of 1 to 2 or 3 times the estimated total blood volume in addition to the original transfusion. Cross-circulation was discontinued when the blood pressure declined to 30 mm. Hg and the dog was virtually in terminal shock. The period of cross-circulation was shorter in the control series than in the liver-perfused group because of the rapid decline and early death. Seven of the 9 dogs died within 12 hours after starting cross-circulation. Some of these dogs had severe bloody diarrhea. One dog died in 18 hours and one dog (LT 26) survived 1 day. The latter dog might have lived this long without cross-circulation, because the [illegible]

fusion period of decline in blood pressure to the level selected for beginning cross-circulation was exceptionally long (3¾ hours).

The liver-perfused dogs (Table II) exhibited several interesting phenomena. "Taking-up," which was as active as in the controls for the first hour or two of cross-circulation, gradually slowed up or disappeared (Table III), an index, we believe, of the recovery of tone in the peripheral vascular system. The blood pressure, which at first was difficult to maintain above 70 to 80 mm. Hg, climbed steadily and eventually stabilized at or above 100 mm. Hg. After some 5 hours of cross-circulation, marked clinical improvement was evident from the return of alertness, restlessness, quick response to environmental stimuli, and the excretion of urine. At this stage cross-circulation was discontinued. After the wounds were

TABLE I

The effect of vivi-perfusion via the femoral vein (controls)

Dog. no.	Dog weight	Volume bled	Shock duration	Best B.P. response to transfusion	Period* of relapse into shock	B.P. at start of cross-circulation	Duration of cross-circulation	Survival
	kgm.	*ml.*	*hrs.*	*mm. Hg*	*hrs.*	*mm. Hg*	*hrs.*	
LT 1	8	400	2½	90	¼	65	2	0
LT 3	10	650	7	80	¼	60	¾	0
LT 6	19	800	3	95	¾	60	4	0
LT 19**	22	1,100	4	85	1	60	7	18 hours
LT 22**	12	450	3	80	1	50	6¼	0
LT 26**	23	780	3½	110	3¾	60	1¾	1 day
LT 27	21	1,400	5	80	¾	45	¼	0
LT 28	24	900	3½	80	½	60	5½	0
LT 29	21	1,150	4½	70	0	30	3	0
			(Average 4)				(Average 3½)	

* Period between end of transfusion and start of cross-circulation.
** Splenectomy done 1 to 3 weeks earlier.

TABLE II

The effect of vivi-perfusion via the splenic vein (liver-perfused dogs)

Dog no.	Dog weight	Volume bled	Shock duration	Best B.P. response to transfusion	Period* of relapse into shock	B.P. at start of cross-circulation	Duration of cross-circulation	Survival	Remarks
	kgm.	*ml.*	*hrs.*	*mm. Hg*	*hrs.*	*mm. Hg*	*hrs.*	*days*	
LT 4	18	750	3	60	½	75	6	+ (1)	Decorticate after drop in B.P. following ligation of splenic pedicle at end of experiment
LT 7	16	525	2½	80	1	50	1	0	
LT 8	20	400	4	100	3¼	65	4¼	+ (7+)	Sacrificed in good health
LT 18	19	550	2	70	0	60	8¾	+ (7+)	Sacrificed in good health
LT 20	20	700	4½	80	¼	50	6	+ (3)	
LT 21	21	800	5½	110	1	60	5½	+ (7+)	Sacrificed in good health
LT 23	24	1,000	3¼	100	½	50	6	+ (2)	
LT 24	19	1,000	3½	100	1	40	5½	+ (2)	Severe pulmonary congestion at autopsy
LT 25	21	1,100	7¾	70	¼	50	3½	+ (2)	? cerebral damage during periods of accidental blood loss during experiment
			(Average 4)				(Average 5½)		

* Period between end of transfusion and start of cross-circulation.

TABLE III
"Taking-up" during vivi-perfusion

	Dog no.	Blood added during each hour of cross-circulation (*ml.*)											
		1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.	7th hr.	8th hr.	9th hr.	Total	Total	Survival
											ml.	*ml. per kgm.*	
Controls	LT 1	600	1,800								2,400	300	0
	LT 3	1,650									1,650	165	0
	LT 6	200	400	200	300						1,100	58	0
	LT 19	200	100	100	400	300	200	300			1,600	73	18 hrs.
	LT 22	1,200	0	0	0	200	0	0			1,400	117	0
	LT 26	1,200	1,050								2,250	98	1 day
	LT 27	1,800									1,800	86	0
	LT 28	450	600	200	200	0	750				2,200	92	0
	LT 29	1,500	500	500							2,500	119	0
											Average:	123	
Liver-perfused	LT 4	400	200	200	0	300	0				1,100	61	+
	LT 7	3,550									3,550	222	0
	LT 8	1,400	400	200	100	0					2,100	100	+
	LT 18	800	550	200	0	200	200	0	200	400	2,550	134	+
	LT 20	200	200	100	100	100	100				800	40	+
	LT 21	600	200	200	300	0	0				1,300	62	+
	LT 23	900	400	200	300	0	0				1,800	75	+
	LT 24	200	200	0	0	0	0				400	21	+
	LT 25	400	600	200	0						1,200	57	+
											Average: (survivors only)	69	

repaired, the dogs were removed from the operation table, appeared strong, walked, and drank water.

Of 9 dogs, 8 survived 1 or more days, which is considered survival from the point of view of the shock state. Dog 7, which died 1 hour after beginning cross-circulation, showed such rapid and unabated "taking-up" as to indicate the onset of terminal shock before liver repair could be achieved. No precautions against infection were observed during the experiments and no after-care was provided for the surviving animals. Bloody diarrhea was observed in 3 dogs. The blood NPN level was normal several days after the experiment in the 2 dogs in which this determination was made. One dog, which died after 48 hours, showed bilateral pneumonia. Three were sacrificed after 7 days. Another, which developed a temporary severe hypotension when the splenic pedicle was tied upon discontinuing the perfusion, became decorticate and, though not in shock, died some 28 hours later. In unanesthetized dogs, gross evidence of brain damage does not appear during the interval of these experiments at blood pressure levels of 30 mm. Hg or higher. But even momentary drops below this pressure result in respiratory failure and signs of decortication. Such drops occasionally occurred in these experiments. Their immediate correction restored normal respiration, but if signs of decortication persisted, the survival period after recovery from shock did not last more than 1 to 2 days.

Data are given in Table IV showing the effects of cross-circulation on the blood concentration values of 4 metabolites. It will be observed that the blood lactic acid, which has already been reduced by the original transfusion (4), is still further reduced by cross-circulation. But since the magnitude of the reduction is equally great in both series, it may be concluded that the lactic acid level bears no relation to the outcome of the shock state. A similar conclusion was reached from other data (4). The fall in blood glucose that occurs after the original transfusion is not alleviated by cross-circulation. The sustained glucose level of the donor in the liver-perfused series indicates that the demand on the donor animal for glucose from the liver-perfused recipient is not so great as from the control recipient. The amino acid level in the liver-perfused series is somewhat reduced [illegible]

TABLE IV

Analyses of blood from the shocked dog before and during vivi-perfusion, in liver-perfused dogs and in controls

Each figure is an average of all experiments. The number of determinations represented in the average is given in the right upper corner of each box. The figure in parenthesis is the average value for the analyses of samples of the donors' blood, taken at the same time as the recipients'.

		Normal	Shock: Before transfusion	Shock: After transfusion	Cross-circulation: 0–4 hrs.	Cross-circulation: 4–8 hrs.
Lactic Acid (*mgm per cent*)	liver-perfused dogs	[9] 28.5	[5] 89.0	[5] 71.5	[5] 37.0 (35.0)	[4] 29.0 (43.0)
	controls	[9] 28.5	[3] 85.5	[5] 54.5	[5] 40.5 (38.5)	[2] 30.0 (33.0)
Glucose (*mgm. per cent*)	liver-perfused dogs	[8] 109	[4] 102	[4] 87	[4] 86 (95)	[4] 70 (141)
	controls	[8] 109	[3] 135	[3] 88	[5] 62 (71)	[2] 67 (74)
Amino N (*mgm. per cent*)	liver-perfused dogs	[14] 11.4	[7] 20.7	[5] 19.2	[10] 14.0 (13.4)	[7] 14.5 (16.0)
	controls	[14] 11.4	[5] 16.2	[4] 14.6	[9] 14.4 (11.8)	[3] 18.4 (15.5)
Urea N (*mgm. per cent*)	liver-perfused dogs	[12] 8.9	[5] 22.2	[4] 20.9	[8] 25.8 (15.0)	[5] 25.2 (25.3)
	controls	[12] 8.9	[5] 20.7	[4] 15.2	[9] 17.6 (16.2)	[3] 20.6 (20.2)

circulation, but is uninfluenced in the control series. Urea synthesis proceeds during the shock state and the donor is unable to reduce the blood-urea level. The shifts in the levels of these metabolites affect donor and recipient about equally, with the exception of glucose in the liver-perfused series. There is no evidence from these data that alterations in the blood levels of these metabolites bear any relation to survival or death of the dog in hemorrhagic shock.

DISCUSSION

These experiments demonstrate that vivi-perfusion of the liver for 5 to 9 hours with arterial blood from a donor animal is an effective therapeutic agent for the treatment of hemorrhagic shock which fails to respond to restoration of normal blood volume and to other agents (5). The contribution of the donor animal consists at least in furnishing a sufficient volume flow of arterial blood to the liver. Does the donor animal contribute something more, such as supplies of glucose and other metabolites, electrolytes, enzymes, and detoxifying or equilibrating processes not available to the dog in shock, *e.g.* renal function? Since such additional benefits are also available to the control dog, the specific and crucial service furnished by the donor must be related to what is supplied directly to the recipient's liver. It is the response of the recipient's liver to this service which results after several hours of cross-circulation in the slowing up and disappearance of the "taking-up" phenomenon and the concurrent recovery of peripheral vascular tone, which is evident from the progressive improvement in the hemodynamics and the disappearance of the shock state.

Our data in a previous publication do not contradict or support the inference that a toxic factor, such as the vaso-depressor material of Shorr *et al* (6), can be implicated as the chief factor in the collapse of the peripheral vascular mechanism. The data herewith presented likewise do not allow of such an inference. It should be added, however, that, if such an inference is not permissible, it is equally invalid on the same evidence to conclude that a protective factor, such as a normally secreted humoral product of hepatic origin, may be invoked to account for the stability or recovery of the peripheral circulation—else why should the donor with a healthy liver fail to support the failing circulation of the control animal?

Whether those functions provided by the donor, which are also available to the control recipient, are or are not indispensable accessories to those supplied by liver perfusion remains to be determined.

What is quite clear from the data at hand is that "irreversibility" in hemorrhagic shock under the experimental conditions described is a result of hepatic injury and that the solution of the problem must be directed toward prevention or correction of hepatic injury.[3]

"The interesting facts stand out that a trifling injury (to the liver) due to phosphorus or chloroform can

[3] The relationship between hepatic injury and shock was observed by Whipple and his coworkers (7) who wrote the following:

be tolerated by a dog with no clinical reaction. But if at this time . . . we perform a plasmapharesis of small volume which was previously tolerated by the same dog with little to no intoxication, we immediately precipitate severe or fatal shock. . . . The chloroform or phosphorus causes an injury to many liver cells and these cells are more susceptible to other injurious agents than are normal liver cells. A sudden change in the protein content of the blood which bathes these injured cells will react more unfavorably upon them than upon the healthy and more resistant normal liver cells. These damaged (phosphorus) and then shocked (plasmapharesis) liver cells form substances which are taken up by the blood and carried to all living cells of the body. If these poisonous substances are sufficient in amount, we observe the development of lethal shock. . . .

"We observe in other experiments that cell injury of other organs (kidney, pancreas, and intestine) does not modify the familiar reaction following a moderate exchange. The control and poisoning experiments give similar reactions. This indicates a peculiar relation of the liver cells to the shock reaction associated with plasma depletion."

SUMMARY AND CONCLUSIONS

Eight of the 9 dogs in hemorrhagic shock irreversible to transfusion recovered after perfusion of the liver by cross-circulation via the splenic vein with a healthy donor dog. Seven of 9 dogs similarly treated, except that the donor's blood entered the femoral instead of the splenic vein, did not survive.

These observations demonstrate (*1*) that liver damage is intimately related to the collapse of the peripheral vascular mechanism in advanced hemorrhagic shock, (*2*) that restoration of the integrity of the liver is necessary for recovery of function of the peripheral vascular system in advanced hemorrhagic shock, and (*3*) that restoration of this aspect of liver function can be accomplished by providing adequate blood flow from a donor dog to the liver, even after "irreversibility" to transfusion has been demonstrated.

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BIBLIOGRAPHY

1. Frank, Howard A., Seligman, Arnold M., and Fine, Jacob, Traumatic shock. XIII. The prevention of irreversibility in hemorrhagic shock by vivi-perfusion of the liver. J. Clin. Invest., 1946, *25*, *22*.
2. Karr, W. G., A method for the determination of blood urea nitrogen. J. Lab. and Clin. Med., 1924, *9*, *329*.
3. Gibson, J. G., 2nd, Seligman, Arnold M., Peacock, Wendell C., Fine, Jacob, Aub, Joseph C., and Evans, Robley D., The circulating red cell and plasma volume and the distribution of blood in large and minute vessels in experimental shock in dogs measured by radioactive isotopes of iron and iodine. J. Clin. Invest., 1947, *26*, *126*.
4. Seligman, Arnold M., Frank, Howard A., Alexander, Benjamin, and Fine, Jacob, Traumatic shock. XV. Carbohydrate metabolism in hemorrhagic shock in the dog. J. Clin. Invest., 1947, 26, 536.
5. Frank, Howard A., Seligman, Arnold M., and Fine, Jacob, Traumatic shock. X. The treatment of hemorrhagic shock irreversible to replacement of blood volume deficiency. J. Clin. Invest., 1945, 24, 435.
6. Shorr, Ephraim, Zweifach, Benjamin W., and Furchgott, Robert F., On the occurrence, sites, and modes of origin and destruction of principles affecting the compensatory vascular mechanisms in experimental shock. Science, 1945, 102, 489.
7. Whipple, G. H., Smith, H. P., and Belt, A. E., II. Shock as a manifestation of tissue injury following rapid plasma protein depletion. Am. J. Physiol., 1920, 52, 72.

TRAUMATIC SHOCK. XV. CARBOHYDRATE METABOLISM IN HEMORRHAGIC SHOCK IN THE DOG[1]

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Traumatic shock may be regarded as a process of rapid biologic disintegration resulting from progressive tissue anoxia consequent to peripheral circulatory failure. This concept explains the recent growth of interest in the intermediary metabolic derangements in shock, which have been found to be severe, involving many enzyme systems concerned with protein and carbohydrate metabolism. Since the liver is a master organ in this respect, it deserves close scrutiny. That this organ should suffer at least as severely as any organ in shock is suggested by the sharp reduction in volume and velocity of flow through the portal system (1, 2) and by the observed reduction of liver excretory functions early in shock (3). Moreover, the recent demonstration that cross-circulation of the dog in hemorrhagic shock from a donor dog via the liver is capable of preventing the onset of irreversibility to transfusion (4) and of curing such irreversibility when established (5) indicates that liver damage plays a primary role in the shock phenomenon. It is, therefore, natural that a relationship should be sought between metabolic derangements in large part normally under liver control and the progressive deterioration characteristic of the late stages of the shock syndrome when therapy is no longer effective.

The metabolism of pyruvic and lactic acids and glucose shows abnormalities in shock (6, 7). In shock the rise in blood pyruvic acid and the simultaneous and usually disproportionately greater rise in lactic acid, producing an increase in the lactic to pyruvic acid ratio, are due to anoxia. Even though changes in blood concentration of these metabolites are not specific manifestations of traumatic shock, since they occur also in fever, following vigorous exercise, epinephrin injection, and in other conditions, a change in the L/P ratio is considered evidence of an abnormal carbohydrate metabolism as, for example, in thiamine deficiency (8). It was considered necessary to explore the possible relationship between spontaneous and induced shifts in the blood concentration of these substances and the progressive deterioration in hemorrhagic shock leading to the development of irreversibility to transfusion.

This communication reports the results obtained from a study of tolerance curves of glucose, lactic and pyruvic acids administered intravenously in various stages of hemorrhagic shock, both before and after transfusion.

METHOD

Irreversible hemorrhagic shock was produced in morphinized dogs by a technic described elsewhere (4). The injection of compounds was followed by arterial blood sampling every 15 minutes for 1 hour thereafter. Tolerance curves were obtained before hemorrhage, during hemorrhagic shock at a constant blood pressure of 30 mm. Hg, and after transfusion of all shed blood. Pyruvic acid[2] (Merck) was given as the sodium salt (neutralized to phenolphthalein) in doses of 0.5 to 1 gram for each tolerance test. Lactic acid (Merck, 85 per cent pure) was given as the sodium salt (neutralized to phenolphthalein) in 1-gram doses.

Blood pyruvic acid was determined by the method of Bueding and Wortis (9), blood lactic acid by the method of Barker and Summerson (10), total amino acid nitrogen by the colorimetric method of Frame, Russell, and Wilhelmi (11). Blood glucose was determined by the method of Folin (12).

RESULTS

1. *Blood levels of lactic, pyruvic, and total amino acids in hemorrhagic shock*

A. In 4 dogs (M–22, 23, 27, 28) there was a progressive rise in blood concentration of all 3 acids before therapeutic transfusion. The rise in lactic acid was steeper than that in pyruvic acid

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] The pyruvic acid was distilled within 1 week prior to the time of injection.

with a resulting 2- to 4-fold elevation of the lactic/pyruvic acid ratio. Figures 1 and 2 give the data for dogs M–23 and M–27, which are typical.

B. In the same 4 dogs after therapeutic transfusion, which was ineffective for survival, the lactic acid level fell proportionately more than the pyruvic acid level, with a return of the L/P ratio to near normal. The amino acid level did not change. In dog M–27, observed for 5 hours after transfusion, a secondary rise in lactic and amino acid occurred after the blood pressure had again fallen below 60 mm. Hg.

2. *Lactic and pyruvic acid blood levels following epinephrin injection in normal dogs*

A continuous intravenous injection of epinephrin hydrochloride (3 mgm. per kgm. per min.) in a volume of 2 to 4 ml. of 0.85 per cent sodium chloride solution was performed in 3 normal dogs (M–15, 38, 39). The lactic and pyruvic acid levels rose promptly in all 3 dogs and the L/P ratio rose in 2 dogs. The lactic acid concentration leveled off after one hour in 2 dogs, but continued to rise in the third dog (M–38), which went into shock during the injection (2 hours) and died 15 minutes after discontinuance of the intravenous drip. In 2 of 3 dogs, elevation of the blood glucose level was followed by a fall to below the initial concentration during the second and third hours. The third dog (M–15) maintained an elevated blood glucose level throughout the experiment which lasted 3 hours. All dogs showed systolic blood pressure levels of about 200 mm. Hg during the epinephrin drip. Dog M–15 survived; dog M–39 (Figure 3), whose systolic blood pressure was 100 mm. Hg after discontinuance of the injection, died 12 hours later.

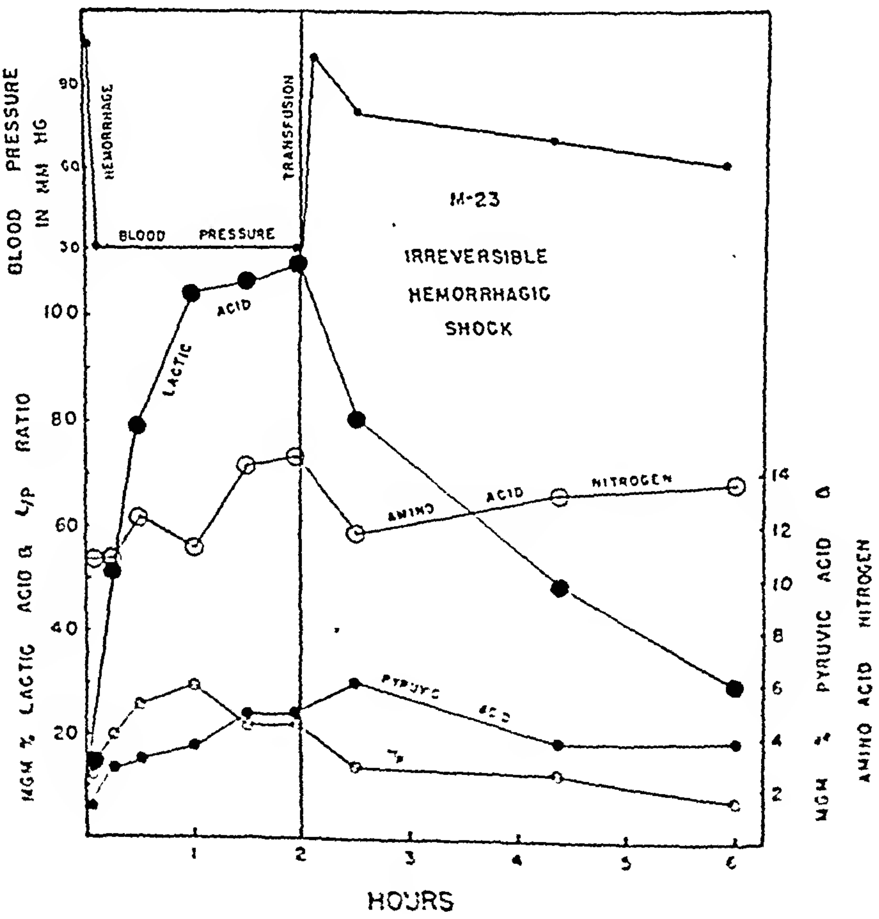

FIG. 1.

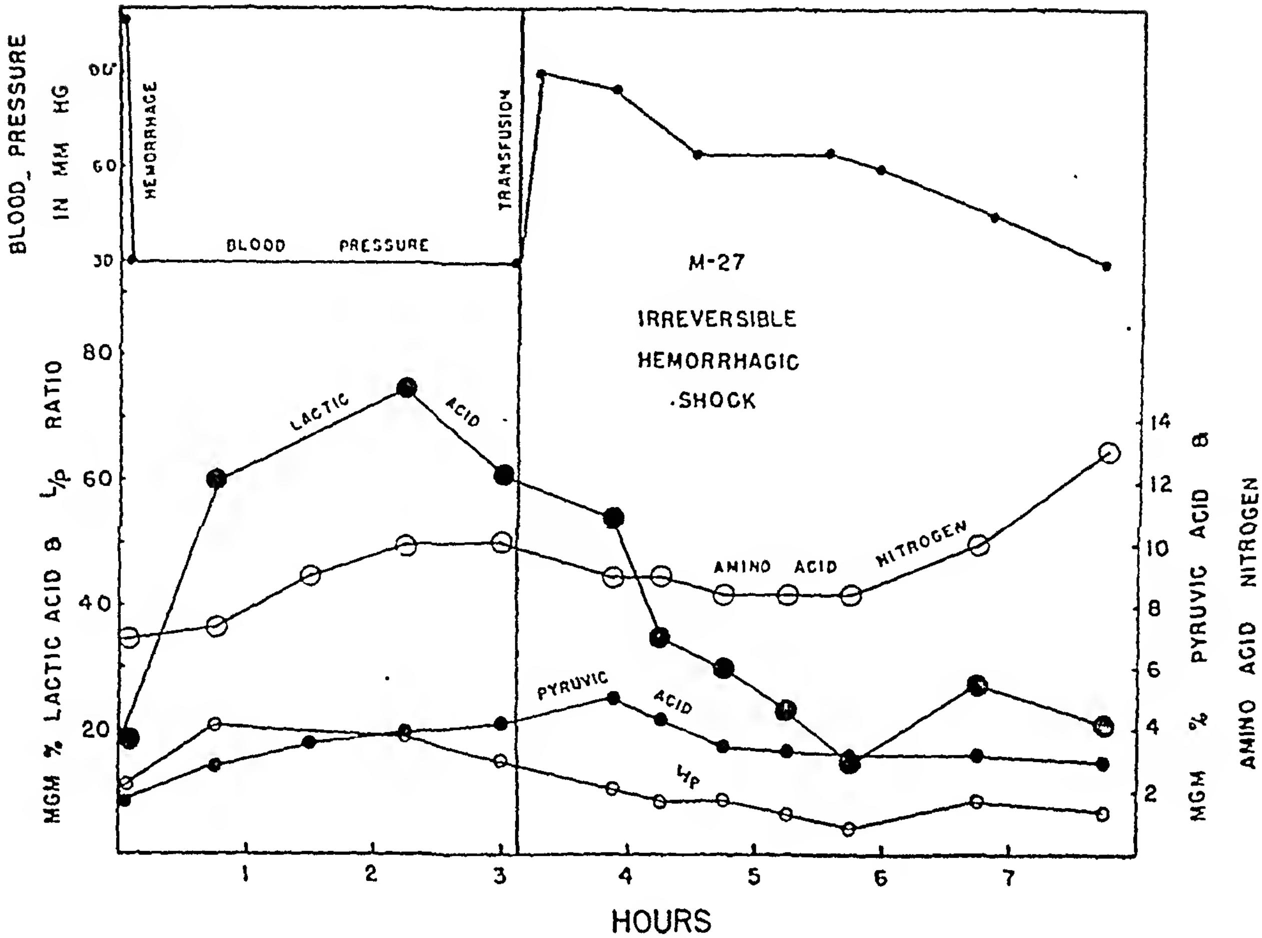

Fig. 2.

The changes in blood lactic and pyruvic acids in these experiments were not unlike those observed during vigorous exercise (8), but of a lower order of magnitude than was observed in hemorrhagic shock.

Comment: The changes in blood lactic and pyruvic acids in shock, therefore, are not referable entirely to epinephrin secretion or to the blood pressure level.

3. *Blood levels of lactic and pyruvic acids in hemorrhagic shock in adrenalectomized dogs*

To determine the relationship of the blood levels of these acids in shock to total adrenal secretion, unilateral adrenalectomy was followed in 2 weeks by removal (under local anesthesia) of the second adrenal. Immediately thereafter hemorrhagic shock was induced. Poor tolerance to hemorrhage and poor response to transfusion were observed. In 5 dogs (M–36, 37, 40, 41, 42) so studied (Figure 4, M–36) though the blood glucose fell, prompt rises in lactic and pyruvic acid and in the L/P ratio similar to those noted above (Section 1) were observed. The lactic and pyruvic acid levels remained elevated after transfusion.

Comment: These changes in shock, therefore, cannot be attributed to epinephrin or cortical hormone secretion. It also appears that they bear no relationship to the level of blood glucose.

4. In vitro *experiments*

To determine to what extent the observed shifts in lactic and pyruvic acids may reflect intravascular changes apart from those due to the activity of tissues other than blood, *in vitro* experiments were made as follows: 650 mgm. of glucose were added to 260 ml. of sterile arterial heparinized blood drawn under oil, with a resulting rise in glucose concentration from 70 mgm. per cent to 330 mgm. per cent. The blood was kept at 37.5° C. under oil for 2 hours and sampled at 20-minute intervals. It took on the color of venous blood during the period of observation. The glucose concentration remained unchanged but the L/P

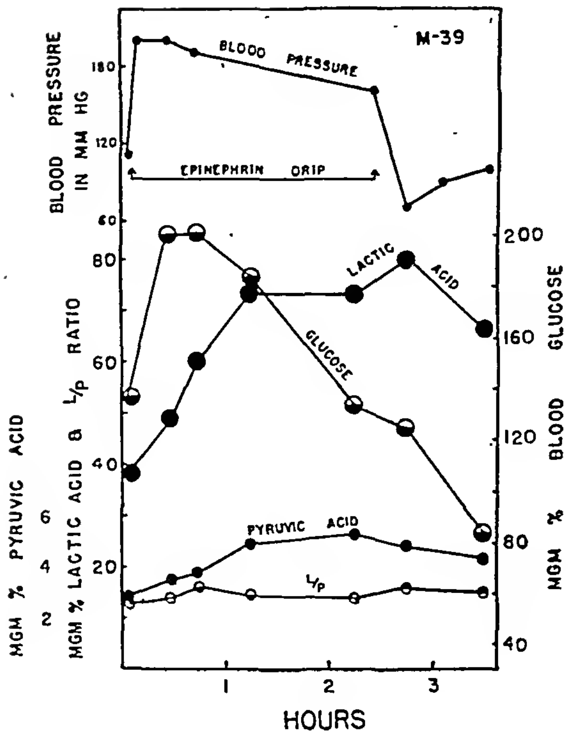

FIG. 3.

ratio rose slightly, though significantly, in 2 hours, in part at least because of a fall in the pyruvic acid, a phenomenon not observed in shock. The

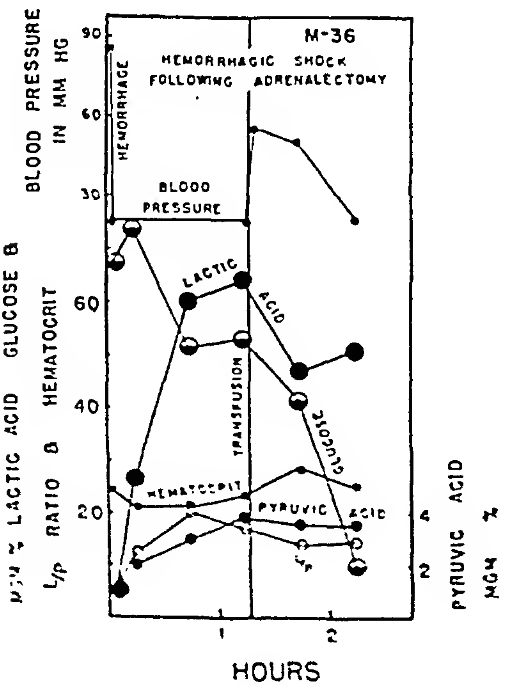

FIG. 4.

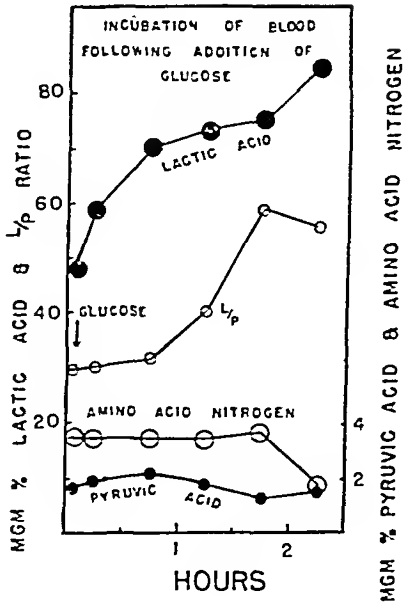

FIG. 5.

changes in lactic acid, pyruvic acid, and amino acid concentration are shown in Figure 5.

One hundred fifteen mgm. pyruvic acid neutralized with NaOH was added to 300 ml. arterial heparinized blood drawn under oil and kept at 37.5° C. The pyruvic acid concentration rose from 4.8 mgm. to 32 mgm. per cent immediately following the addition of pyruvic acid and dropped to 30 mgm. per cent in 1.5 hours. There was a concomitant rise in lactic acid, presumably derived

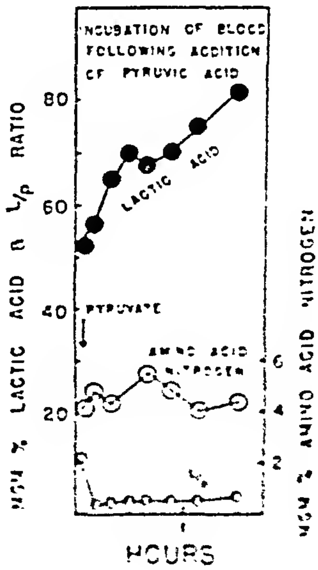

FIG. 6.

from the added pyruvate through the action of the red cells, Figure 6.

Comment: These observations indicate that a decline in blood oxygen content *per se* does not account for any substantial fraction of the rise in the blood lactic acid in shock.

5. *Glucose tolerance curves in hemorrhagic shock*

Seven dogs (M–16, 29, 30, 33, 34, 47, 48), in which irreversible hemorrhagic shock was produced, were given 1 gram per kgm. of glucose intravenously before hemorrhage, again during shock, and again following transfusion. Glucose, lactic and pyruvic acid levels in arterial blood were determined at 15-minute intervals after each injection.

The disappearance of injected glucose from the blood in 4 normal dogs required more than 1 hour. The disappearance rate during hemorrhagic shock was prolonged, but following transfusion it was near normal, whether the subsequent decline in blood pressure was precipitous or gradual.

Comment: The lactic and pyruvic acid levels characteristic of shock appeared not to be influenced by the injection of glucose (Figure 7).

6. *Lactic acid tolerance curves in hemorrhagic shock*

Sodium lactate, injected into normal dogs, increased the blood lactic acid concentration from 10 to 30 mgm. per cent at the peak of the rise. The lactate was usually, but not always, completely cleared. In one experiment the arterial blood pH was lowered from 7.4 to 7.2 (glass electrode). No significant alteration in blood pyruvic acid occurred.

Following the injection of lactate in hemorrhagic shock (Figure 8, M–19), the resulting superimposed rise in the blood lactic acid level was no greater than that following the injection of lactate in the normal dog. The shocked animal was able to clear the blood of injected lactate about as well as the normal dog.

Following transfusion, injected lactate was cleared from the blood at a normal or greater than

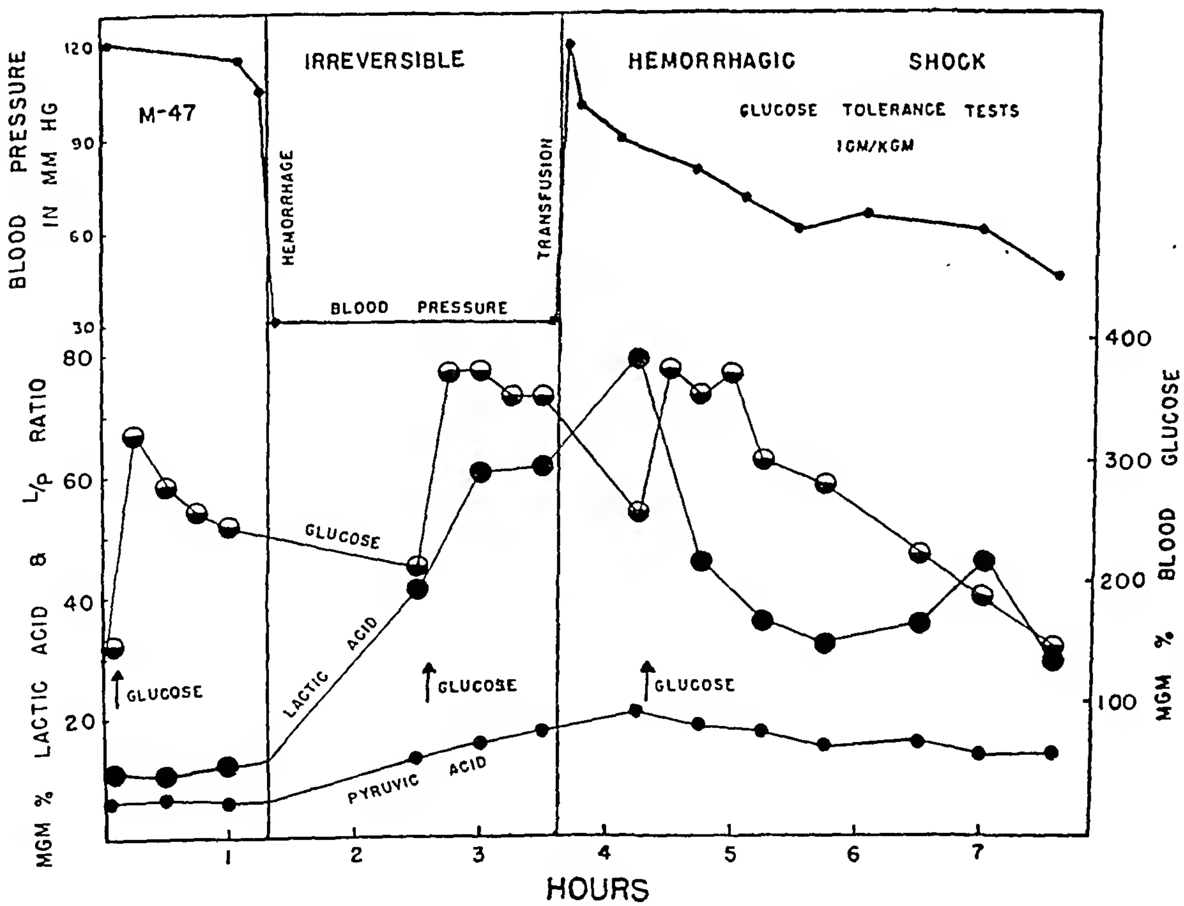

Fig. 7.

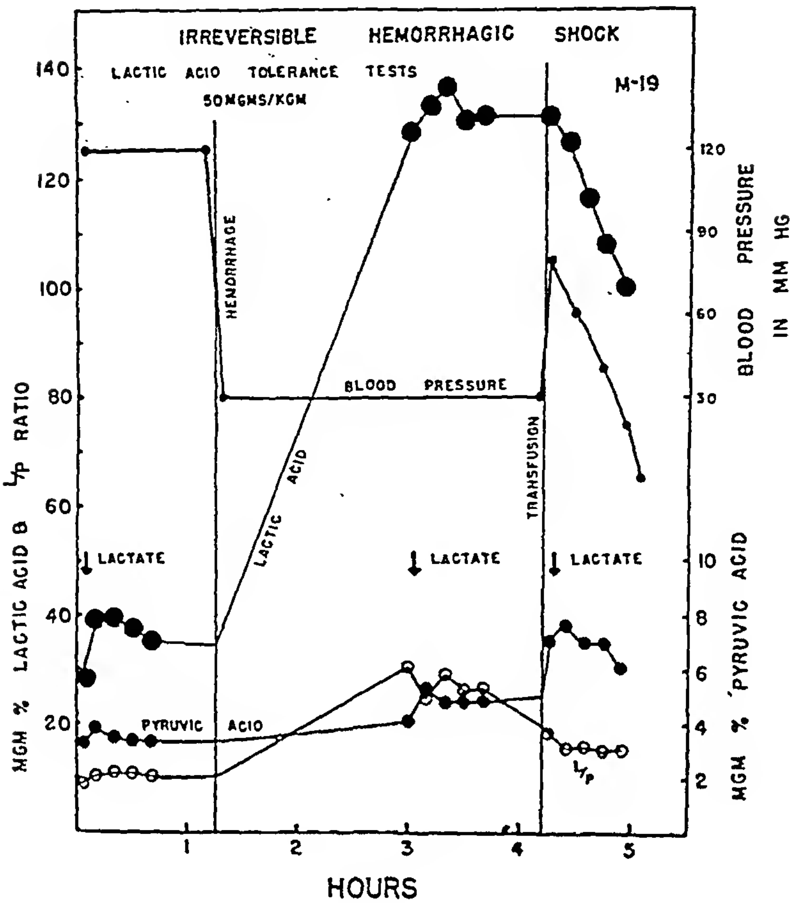

Fig. 8.

normal rate. In one experiment (Figure 8, M–19) the expected fall in blood lactic acid level was so rapid as to mask completely the increase due to injected lactate, even though a transfusion was ineffective and was followed by a rapid decline in blood pressure.

Comment: Since injected lactate is cleared from the blood in hemorrhagic shock at a normal rate even when the animal is dying after an ineffective transfusion, the deterioration of the organism in shock cannot be accounted for by the failure of enzyme systems to catabolize this carbohydrate intermediate. The foregoing data also suggest that an increase in the rate of lactic acid production is not the sole determinant of the level achieved. An alteration in the equilibrium between lactate and pyruvate or other carbohydrate intermediates may exist and be due in part to impairment of volume and velocity of blood flow rather than to damage to enzyme systems.

7. Lactic acid tolerance in acidosis

To learn whether the concentrations of lactic and pyruvic acids are determined by acidosis prevailing in shock, 1-gram doses of neutralized lactic acid were injected intravenously into normal dogs before and after the production of acidosis by intravenous hydrochloric acid. In one dog (M–53, Figure 9) the lactic acid tolerance curve following the injection of 1 gram of lactate was the same before and 10 minutes after the injection of 25 ml. of a 10 per cent solution of hydrochloric acid.

In another dog (M–52), 1 gram of lactate raised the arterial pH from 7.4 to 7.6. In the course of 30 minutes, 250 ml. of 0.1 N HCl were then injected. During the injection the blood pressure dropped to 70 mm. Hg, then to 30 mm. Hg and the arterial blood pH dropped to 7.2, but the blood lactic acid rose only 10 mgm. per cent. Death occurred a few minutes later. In this experiment

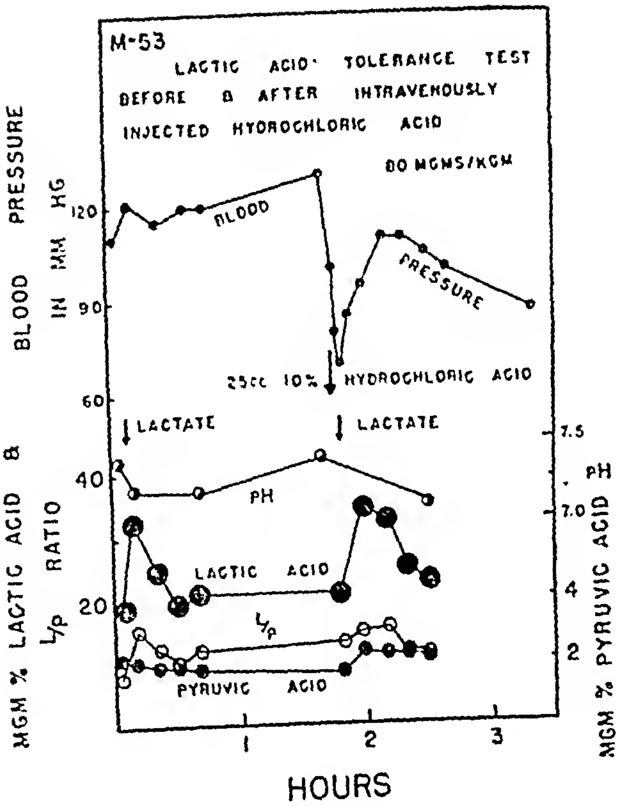

FIG. 9.

8. *Pyruvic acid tolerance in normal dogs*

Following the injection of pyruvic acid (0.05 to 0.1 gram per kgm. dog) neutralized to phenolphthalein, the return to the basal value occurred within 45 minutes in 13 of 17 normal dogs. The original level was not reached in this time in 4 dogs. The increase following the injection of pyruvate varied from 0 to 1.0 mgm. per cent. Concomitant lactic acid determinations showed very slight rises or no effect, but drops in lactic acid of 5 to 10 mgm. per cent below the starting level were noted in nearly each case at the end of 45 minutes (Figure 11). In 6 of 9 dogs the amino acid nitrogen was also depressed 2 or more mgm. per cent 45 minutes after the injection of pyruvic acid in 6 of 9 dogs.

9. *Pyruvic acid tolerance in hemorrhagic shock cured by transfusion*

In 4 of 7 dogs cured of hemorrhagic shock by transfusion, the pyruvic acid tolerance curves were the same before and after transfusion as in the nor-

acidosis more severe in degree than occurs in hemorrhagic shock did not produce so great an elevation of the blood lactic acid level as is found in shock.

In a third dog (M–51, Figure 10) repeated injections of 1 N hydrochloric acid caused a steady fall in the CO_2 combining power of the blood and in arterial blood pH. At the same time there was a steady rise in blood lactic acid, while pyruvic acid changed very little. Though the L/P ratio rose, it remained within normal limits. During this time the injection of 0.5 gram of lactate produced a normal tolerance curve (superimposed on the steadily rising blood lactic acid level) similar to that observed in hemorrhagic shock (Figure 8). The dog developed circulatory collapse and died when acidosis became severe.

Comment: It appears that the dog in acidosis metabolizes lactic acid as well as the normal or shocked animal. The rise in the blood levels of pyruvate and lactate (see Figures 9 and 10), following the production of a severe grade of acidosis, is not of the magnitude observed in hemorrhagic shock. The changes in these acids seen in hemorrhagic shock are not determined by the concurrent acidosis.

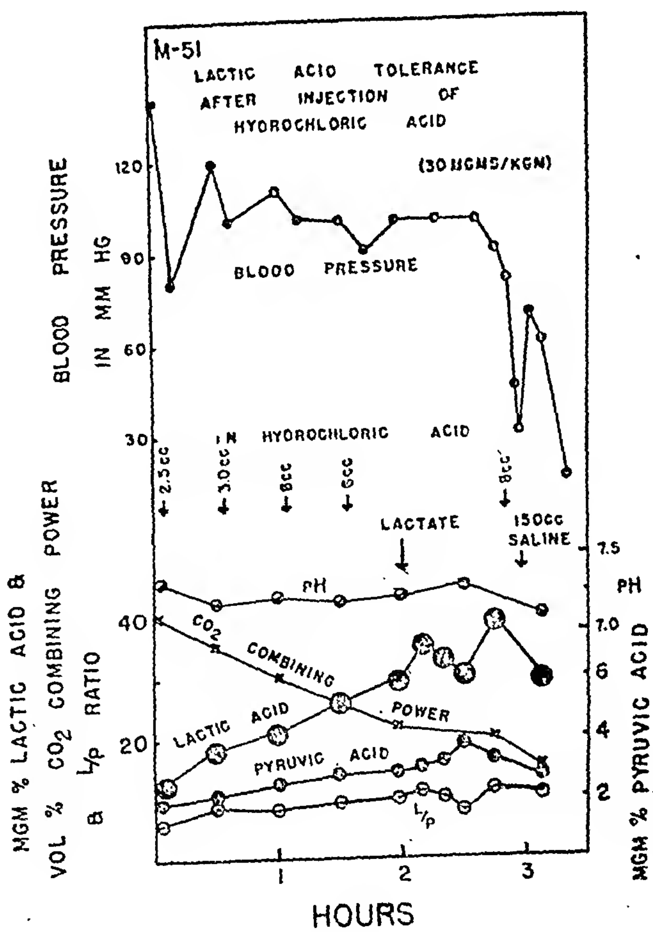

FIG. 10.

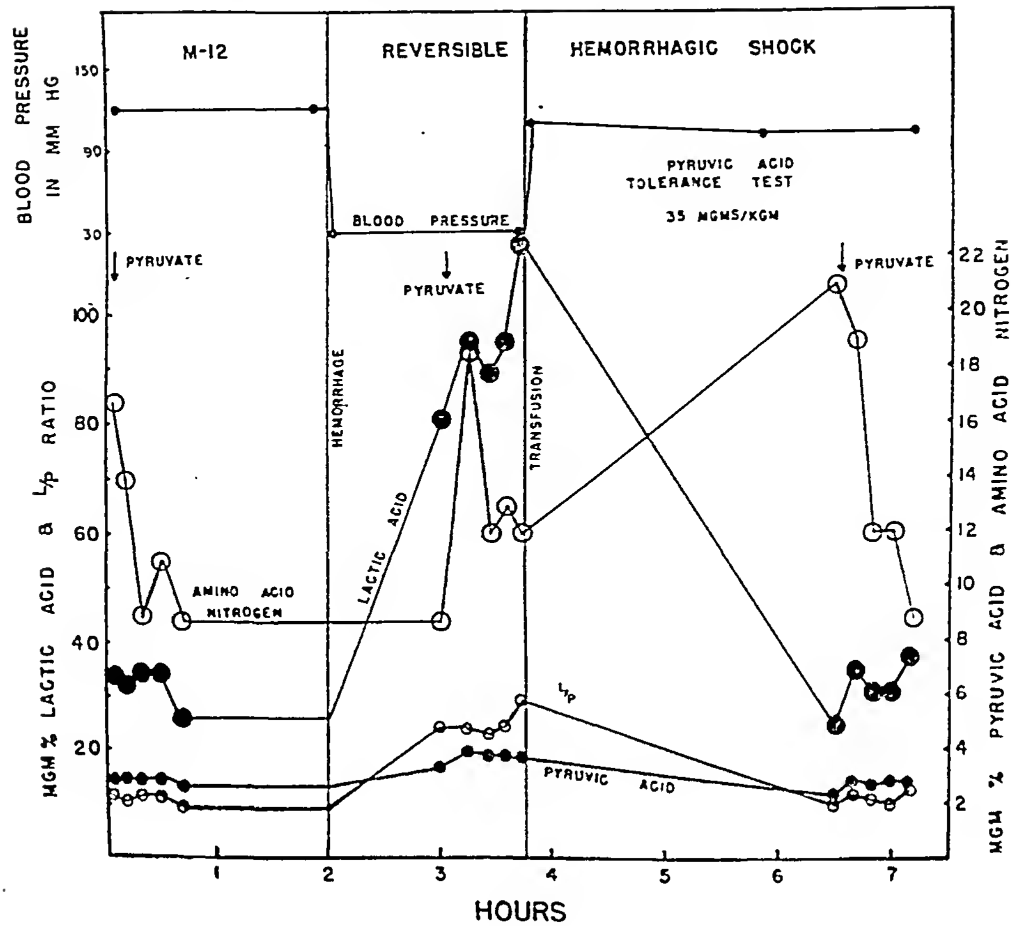

FIG. 11.

mal or preshock stage, although the preinjection levels of bloods pyruvate were higher during the shock state than before shock was instituted. In the remaining 3 dogs the last point of the tolerance curves during shock before and after transfusion were slightly higher than the preinjection level (Figure 11). The maximum rise in pyruvic acid during shock was in some instances slightly higher than in the normal. In some experiments evidence of lactic acid production from injected pyruvate during shock was suggested by the rapid rises in lactic acid levels immediately after the injection of pyruvate. Subsequent depressions in amino acid levels were observed. The usual rise in the L/P ratio during shock and its return toward normal following transfusion was in no way altered by pyruvate injections.

Comment: Elevation of the pyruvic acid level in shock is not due to inability of the organism to clear the blood of pyruvic acid. The significance of the drop in amino acid nitrogen following pyruvate injection will be discussed in a subsequent publication.

10. *Pyruvic acid tolerance in hemorrhagic shock not remediable by transfusion*

Similar results were obtained in 3 of 9 dogs in hemorrhagic shock which did not respond to transfusion (M–9, Figure 12). In 5 dogs, a somewhat poorer clearance occurred during shock than in the normal or in the post-transfusion stage. One dog (M–21) showed very poor clearance before and after transfusion as compared to the normal. Although this dog received twice the usual dose of pyruvate, 2 others which received the double dose showed only a slightly poorer clearance in the shock stage than in the normal. The injection of pyruvate in the n[illegible] [illegible] in some [illegible] One normal d[illegible]

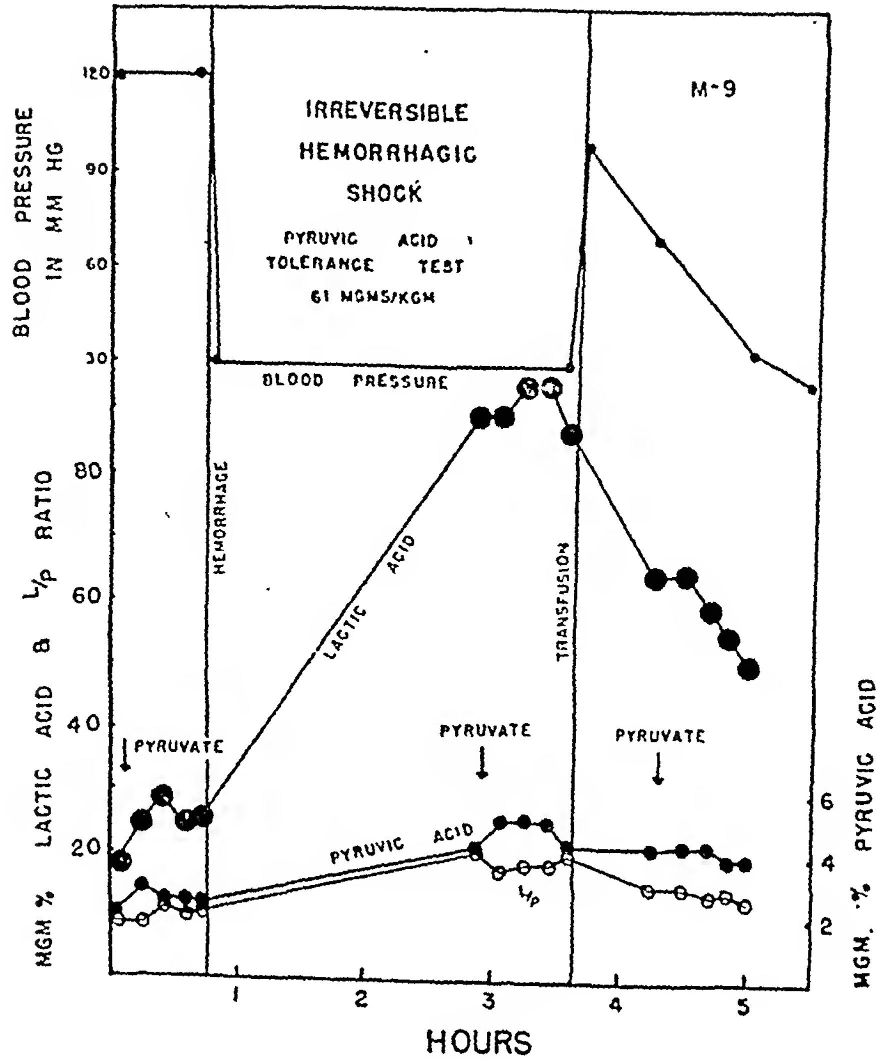

FIG. 12.

into shock for a considerable time following the injection of a double dose. Figure 12 shows that the rise of lactic acid during shock and the fall following transfusion are not altered by the injection of pyruvic acid. Depression of the amino acid level was noted among these dogs as well as in the previous group (*cf.* Figure 11 with Figures 1 and 2).

Comment: It is concluded that non-toxic doses of pyruvate are cleared by the dog in shock somewhat less well than in the normal before transfusion and about as well as normal after transfusion, whether reversible or irreversible to transfusion. These results suggest that the slightly decreased clearance in the pre-transfusion stages is due to decreased blood flow, particularly through the liver, rather than to any specific damage to the enzyme systems involved, for if damage to enzyme systems were responsible for defective clearance, one would not expect so prompt a recovery of clearance function following transfusion, and one would expect a disparity between the results in irreversible and reversible shock.

DISCUSSION

It has been demonstrated in traumatized rats (13) that the accumulation in the blood of lactic acid, inorganic phosphate, and phosphopyruvic acid occurs simultaneously with a progressive exhaustion of energy reservoirs in tissues (14), particularly in liver and brain. A correlation has been said to exist between survival and the magnitude of the original rise in the blood level of these intermediary metabolites as well as in the speed of their return to normal values (13). If the blood concentrations of these metabolites reflect the extent of the biochemical lesion in the tissues, a study of their behavior in response to therapeutic transfusion in hemorrhagic shock in dogs might provide a key to the nature of the irreversible lesion and at the same time demonstrate whether they are essential components of the lethal process or

merely concurrent phenomena of secondary importance.

Russell *et al* (7) concluded that since eviscerated (liverless) rats do not show elevations in the L/P ratio comparable to those in non-eviscerated rats or eviscerated shocked rats, peripheral anoxia rather than liver failure is largely responsible for the increase in the L/P ratio. Dogs in hemorrhagic shock show a progressive increase in lactate and a fall in glucose in the venous as compared to the arterial blood, suggesting an increased conversion of glucose to lactate caused by the anoxia of peripheral vascular failure (15). Our experiments are consistent with these conclusions. They indicate further that the actual level of blood lactate achieved is controlled by a factor other than the mere rate of production of lactic acid.

The rise in the L/P ratio in hemorrhagic shock results from the predominance of anaerobic over aerobic carbohydrate metabolism, especially in muscle (16), in the presence of peripheral circulatory failure. In this situation energy is derived largely from glycolysis, whose end product is lactic acid. This is much less efficient than aerobic oxidation since 8 to 14 times as much substrate is required to yield the same amount of energy (17).

In spite of the rise in lactic and pyruvic acids and the increase in the L/P ratio in shock, additional loads of these acids and of glucose are metabolized almost as well as in the normal state,[8] regardless of the severity of the shock state or the response to transfusion. The return of the L/P ratio to normal following transfusion indicates a return to aerobic metabolism of carbohydrate. Since this is the case whether or not the transfusion is therapeutically effective, it appears that even a temporary improvement of blood flow is capable of restoring the normal rate of carbohydrate breakdown but is not capable of restoring the integrity of the factor responsible for maintenance of normal function of the peripheral vascular system. It follows that the distortion in carbohydrate metabolism, as reflected by the blood changes studied, is probably not a primary factor in peripheral vascular failure. If it were otherwise, death from ordinarily fatal shock would not be prevented by liver perfusion, during which the distortion of carbohydrate metabolism is of the same kind and magnitude as is seen in shocked dogs not perfused or perfused via another route (4). To be sure, our blood analytical data may not disclose persisting metabolic abnormalities in certain vital tissues, whose function, therefore, continues to be adversely affected; for example, the brain stem may suffer from the decrease in oxygen supply (14) in spite of the apparently normal enzymatic behavior suggested by the blood findings. But there is no evidence to indicate that in the circumstances of our experiments any organ apart from the liver is of critical importance with respect to the collapse of the peripheral vascular system in shock, since such organs, including the brain, respond well to transfusion after exposure to a degree of hypotension and hypoxia which the liver cannot tolerate. Since the liver has been shown to be the critical organ with respect to the integrity of the peripheral vascular system in shock, the damage it suffers involves at least another category than that of carbohydrate oxidation.

[8] We do not assume that the disappearance of these metabolites from the blood in shock is necessarily achieved by the mechanism prevailing in the normal state.

SUMMARY

1. Morphinized dogs bled into shock showed rapid elevation of blood lactic acid, pyruvic acid, amino acid nitrogen, and the lactic/pyruvic acid ratio.

2. A rapid return toward normal of blood lactic and pyruvic acids and the lactic/pyruvic acid ratio followed transfusion, whether or not the transfusion was curative.

3. The blood amino acid nitrogen remained elevated following transfusion.

4. A continuous drip of epinephrin produced elevations in blood glucose, but much smaller elevations in blood lactic and pyruvic acids and in the lactic/pyruvic acid ratios than occur in hemorrhagic shock.

5. In adrenalectomized dogs, whose tolerance to hemorrhage was poor, the blood levels of lactic and pyruvic acid and the lactic/pyruvic acid ratio increased even though the blood glucose level fell.

6. Arterial blood under oil kept at body temperature showed a slight increase in lactic acid and the lactic/pyruvic acid ratio.

7. The disappearance of injected glucose from the blood was poor or nil during hemorrhagic shock. Following transfusion (even in irreversible shock) this clearance was normal.

8. The clearance of injected lactate from the blood during shock was normal. The restoration of the elevated lactic acid level to normal by transfusion is such as to mask completely the expected increase in level from injected lactate.

9. Clearance of injected lactate in normal dogs was not altered by acidosis induced by the intravenous administration of mineral acid.

10. In normal dogs the pyruvic acid level in the blood returned to normal within 45 minutes following the injection of pyruvate.

11. The elevated lactic/pyruvic acid ratio in shock was only temporarily altered by injected pyruvate.

12. Dogs in irreversible hemorrhagic shock showed disappearance rates for injected pyruvate similar to the normal in the post-transfusion phase of shock but somewhat poorer in the hypotensive pre-transfusion phase of shock, when doses of pyruvic acid that were not toxic were used (under 0.1 gram per kgm.).

CONCLUSIONS

It is concluded that while hemorrhagic shock may interfere with the functional efficiency of the enzyme systems involved in carbohydrate metabolism, the integrity of the enzyme systems involved in lactic and pyruvic acid metabolism is not seriously damaged, because they show a rapid return to normal function following transfusion. The occasionally lowered tolerance to injected glucose and pyruvic acid noted during the oligemic phase of hemorrhagic shock is due to deficient velocity and volume of blood flow through tissues. The accumulation of these metabolites in the blood results from the combined effects of poor blood flow and an increased rate of production in anoxic tissues.

The development of irreversibility to transfusion in hemorrhagic shock is not due to inability of the liver to metabolize lactic and pyruvic acids. Abnormalities in carbohydrate metabolism, so far as they are reflected in the elevations of lactic and pyruvic acids, are not related to the development of irreversibility to transfusion.

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BIBLIOGRAPHY

1. Blalock, A., and Levy, S. E., Effect of hemorrhage, intestinal trauma and histamine on partition of the blood stream. Am. J. Physiol., 1937, 118, 734.
2. Wiggers, C. J., Opdyke, D. F., and Johnson, J. R., Portal pressure gradients under experimental conditions, including hemorrhagic shock. Am. J. Physiol., 1946, 146, 192.
3. Goldberg, M., and Fine, J., Unpublished observations.
4. Frank, H. A., Seligman, A. M., and Fine, J., Traumatic shock. XIII. The prevention of irreversibility in hemorrhagic shock by vivi-perfusion of the liver. J. Clin. Invest., 1946, 25, 22.
5. Seligman, A. M., Frank, H. A., and Fine, J., Traumatic shock. XIV. The successful treatment of hemorrhagic shock irreversible to transfusion by vivi-perfusion of the liver. J. Clin. Invest., 1947, 26, 530.
6. Engel, F. L., Winton, M. G., and Long, C. N. H., Biochemical studies on shock. I. The metabolism of amino acids and carbohydrate during hemorrhagic shock in the rat. J. Exper. Med., 1943, 77, 397.
7. Russell, J. A., Long, C. N. H., and Engel, F. L., Biochemical studies on shock. II. The role of the peripheral tissues in the metabolism of protein and carbohydrate during hemorrhagic shock in the rat. J. Exper. Med., 1944, 79, 1.
8. Stotz, E., and Bessey, O. A., The blood lactate-pyruvate relation and its use in experimental thiamine deficiency in pigeons. J. Biol. Chem., 1942, 143, 625.
9. Bueding, E., and Wortis, H., The stabilization and determination of pyruvic acid in blood. J. Biol. Chem., 1940, 133, 585.
10. Barker, S. B., and Summerson, W. H., Colorimetric determination of lactic acid in biological material. J. Biol. Chem., 1941, 138, 535.
11. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., Colorimetric estimation of amino nitrogen in blood. J. Biol. Chem., 1943, 149, 255.
12. Folin, O., Two revised copper methods for blood sugar determinations. J. Biol. Chem., 1929, 82, 83.
13. McShan, W. H., Potter, Van R., Goldman, A., Shipley, E. G., and Meyer, R. K., Biological energy transformations during shock as shown by blood chemistry. Am. J. Physiol., 1945, 145, 93.
14. Le Page, G. A., Biological energy transformations during shock as shown by tissue analyses. Am. J. Physiol., 1946, 146, 267.
15. Beatty, C. H., The effect of hemorrhage on the lactate/pyruvate ratio and arterial-venous differences in glucose and lactate. Am. J. Physiol., 1945, 143, 579.
16. Friedemann, T. E., and Barborka, C. J., The significance of the ratio of lactic to pyruvic acid in the blood after exercise. J. Biol. Chem., 1941, 141, 993.
17. Lipmann, F., A Symposium on Respiratory Enzymes. Univ. of Wisconsin Press, Madison, 1942.

STUDIES OF CONGENITAL HEART DISEASE. I. TECHNIQUE OF VENOUS CATHETERIZATION AS A DIAGNOSTIC PROCEDURE[1]

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Recent advances in the surgical treatment of congenital disorders of the heart (1 to 4) have increased the importance of accurate diagnosis. Physical and x-ray signs are not easily understood or interpreted, partly because there is relatively little systematic knowledge of the dynamics of the circulation in patients with congenital heart lesions. In the past it has been difficult to acquire such knowledge. Many of the methods of measuring cardiac output are applied with difficulty, if at all, in patients with congenital heart disease, and certain aspects of the pulmonary circulation have heretofore been almost immune from measurement.

The introduction by Forssman (5) of the cardiac catheter and its practical improvement by Cournand and Ranges (6) have offered a new opportunity for the study of congenital heart disease. By these techniques, pressures may be measured in the great veins, the right auricle and ventricle, and the pulmonary artery. Analysis of blood samples obtained from these areas and from a systemic artery permits the calculation of the cardiac output by the Fick principle. This communication describes the methods as they have been applied to patients with congenital cardiac defects. Subsequent papers will describe results obtained in controls and in patients with various congenital cardiac lesions.

METHOD

Patients with congenital heart disease are admitted to the hospital, preferably for a period of 4 days and 3 nights. Record is made of the history, physical examination, red blood cell count, hemoglobin, hematocrit, vital capacity, and usually the circulation time (cyanide or decholin) and venous pressure. X-ray films are taken and fluoroscopic examination of the heart is carried out. The catheterization routine as developed has been designed specifically for the investigation of congenital heart disease in order to assist in the diagnosis and to contribute additional information relative to the disordered circulation in the presence of the congenital abnormality. The following routine of measuring oxygen consumption, carrying out venous catheterization, and obtaining blood by arterial puncture in sequence instead of simultaneously is not the customary procedure for calculating cardiac output, but has been developed because it allows multiple sampling from each chamber. The advantages of multiple sampling will be dealt with in subsequent communications (7, 8).

At 7:30 on the morning of the third hospital day, the basal metabolic rate is measured and the oxygen consumption calculated. Immediately after this procedure the patient is transferred to the fluoroscopy room for venous catheterization. Although the technique of this procedure has been described in detail by Cournand and his associates (9), certain modifications have been introduced to meet specific problems. The patient lies on a radiolucent mat of sponge rubber, 2 inches thick, on the fluoroscopy table. This aids in maintaining comfort and, hence, in avoiding venous spasm. Ten thousand units of penicillin are administered intramuscularly before and again after catheterization even though there seems to be little risk of producing bacterial endocarditis by this procedure. No medication other than penicillin and novocaine without adrenalin is administered. With aseptic precautions, a No. 8-F or No. 9-F radiopaque catheter,[2] 100 cm. long, with the hole at the tip and with the end curved at an angle of about 45°, is used. The technique of right heart catheterization as described by Cournand *et al* (9) has been followed closely. Once the tip has been introduced into the right auricle and the right ventricle, it is directed upward so that it is deflected from the left wall of the right ventricle, through the pulmonary valve, and into the main stem of the pulmonary artery. Since the curve of the tip of the catheter at this point is to the patient's right, it usually passes easily into the right pulmonary artery. To introduce the catheter into the left branch it

[1] This investigation was aided in part by a grant from the John and Mary R. Markle Foundation and from the Proctor Fund of the Harvard Medical School.

[2] This catheter can be obtained from the [illegible] Catheter & Instrument Corp., [illegible] New York. For this procedure a fairly stiff [illegible] to be far less traumatic than a limp [illegible] custom to heat harden the catheter [illegible] by placing it in a [illegible] dry air oven at 120° C.

is withdrawn to the main stem of the pulmonary artery and is rotated in such a fashion as to turn the curved tip to the patient's left. It may then be directed into the left pulmonary artery. This rotation is sometimes difficult to accomplish since the tip of the catheter is at the end of a horseshoe bend.

Samples of blood from pulmonary "capillaries" are obtained by introducing the catheter as far out as possible into a distal ramification of one of the pulmonary arteries (see Figure 1) in such a fashion as to obstruct the vessel. If the catheter is properly placed, fully oxygenated (arterial) blood may be withdrawn through the lumen of the catheter (7, 10).

Blood samples are withdrawn through the catheter under oil as described by Cournand *et al* (9), x-ray films are taken, and pressures with a Hamilton manometer (11) are routinely recorded at each position of the catheter. Blood samples and pressures are obtained first from the pulmonary artery, next from the right ventricle, and finally from the right auricle and superior vena cava. This

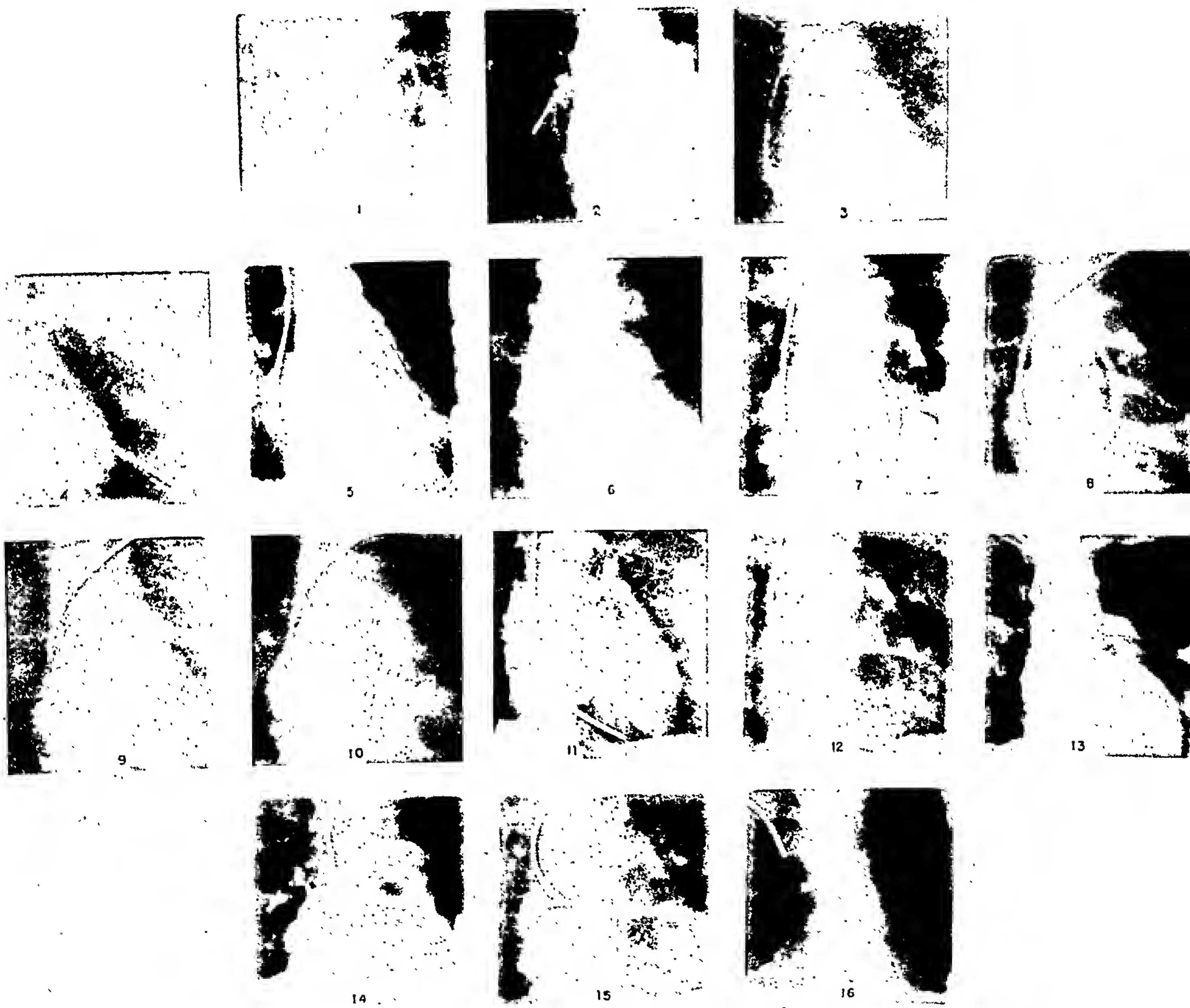

Fig. 1. X-Rays Showing the Catheter in Different Parts of the Heart and Pulmonary Artery

(1) Right and (4) left pulmonary "capillaries"; (2) and (3) right pulmonary artery; (5) and (6) left pulmonary artery; (7) main trunk of pulmonary artery; (8) right ventricle near pulmonary valve; (9) middle of right ventricle; (10) right ventricle near tricuspid valve; (11) lower part of right ventricle; (12) right auricle near tricuspid valve; (13) lower part of right auricle; (14) middle of right auricle; (15) upper part of right auricle; (16) superior vena cava.

order of sampling is followed in order to avoid the dilemma encountered when venous spasm prevents all manipulation of the catheter except its withdrawal. In this way, multiple sampling from each chamber is ensured.

Sites of routine sampling are as follows (see Figure 1):

1. Pulmonary artery.
   a. As far out as possible (for pulmonary "capillary" blood) (7, 10).
   b. Several centimeters beyond bifurcation.
   c. Main stem.
2. Right ventricle.
   a. Near pulmonary valve.
   b. Mid-position.
   c. Near tricuspid valve.
3. Right auricle.
   a. Lower part.
   b. Near tricuspid valve.
   c. Upper part.
4. Superior vena cava.

While it is often not feasible or possible to obtain samples from each of these positions, at least 2 and preferably 3 samples are withdrawn from different parts of each chamber in every case.

Knowledge of the precise location of the tip of the catheter is essential for proper interpretation of data. While an approximation of the location may be made by fluoroscopic observation, it is frequently impossible to ascertain on which side of a valve the catheter tip lies. Under these circumstances, observation of the deflections of the Hamilton manometer before and after the sampling gives precise information regarding the position of the tip of the catheter since the character of the pulse wave and the pressures are quite different in pulmonary artery, right ventricle, and right auricle (7).

Following the advice of Cournand (12), we have not ligated the vein following removal of the catheter. Instead, the skin edges have been approximated with flamed adhesive and a pressure dressing with an elastic bandage has been applied. Usually, little soreness and only local thrombosis of the vein ensue. After 3 days the patient himself may remove the bandage and, in a week, the adhesive strip.

At the conclusion of venous catheterization, a sample of blood is withdrawn under oil from the femoral artery using novocaine as an anesthetic. The oxygen content of each blood sample is determined by the method of Van Slyke and Neill (13). Since at least 10 samples are taken during the procedure, duplicate determinations have been performed only on those samples exhibiting a deviation from neighboring samples.

Clotting of samples is at times troublesome, especially in polycythemic patients. This has been consistently avoidable by injecting about 2 ml. of a 0.05 per cent solution of heparin through the catheter just before sampling.

Oxygen capacity is determined routinely on the arterial and at least one of the venous samples by the method of Van Slyke and Neill (13). Ordinarily the oxygen capacity of different blood samples is essentially the same. The capacity may vary considerably, however, especially in samples of blood with a high hematocrit. Hemoglobin concentrations are, therefore, determined photocolorimetrically on all samples, and those at variance are then corrected by determining the oxygen capacity. Variations in oxygen capacity may thus be detected and the corresponding contents corrected.

DISCUSSION

The procedure described in this report has been formulated with the specific purpose of studying the cardiovascular dynamics in congenital heart disease and of applying the knowledge so obtained to diagnosis.

Calculation of blood flow: The most accurate method at present available for the estimation of cardiac output in man is based on the principle outlined by Fick (14) in 1870. Some of us (15) applied this principle in a group of patients with patent ductus arteriosus in 1941. Since its application in patients with abnormal cardiac communications presents special problems, it seems appropriate to restate the principle and discuss its application in patients with this and other types of congenital heart disease which the catheter method has enabled us to investigate.

Fick (14) derived an equation for the calculation of cardiac output which may be restated as follows:

$$\text{Cardiac output (l. per min.)} = \frac{\text{Oxygen intake (ml. per min.)}}{\text{Arteriovenous oxygen difference (ml. per l.)}}.$$

Similar data derived from determinations of carbon dioxide can be used. Carbon dioxide data have been omitted, however, since the results of these determinations are variable (16). In normal individuals without shunts between cardiac chambers, the calculation is based on the determination of oxygen consumption, oxygen content of arterial blood, and oxygen content of mixed venous blood. The first two determinations are easily obtained. The procurement of well-mixed venous blood is more difficult. In normal individuals, samples from the right auricle and right ventricle may show considerable variation, whereas those taken from the proximal parts of the pulmonary artery show minimal variation and represent acceptable values for mixed venous blood (7).

In patients with congenital heart disease, as will be shown (8), it is frequently impossible to obtain samples of blood that are adequately mixed if these samples are taken near a shunt. Although this detracts from the accuracy of the estimation, nevertheless reasonable approximations of flow through the various chambers may be obtained.

To estimate the blood flow through a shunt, the peripheral blood flow and pulmonary blood flow must be calculated separately. The peripheral blood flow may be calculated in the usual fashion except that the sample of mixed venous blood must be obtained proximal to the shunt. For example, in cases with patent ductus arteriosus, it must be obtained by multiple sampling from the right ventricle; in cases with ventricular septal defect, from the right auricle; and in cases of auricular defect, from the vena cava. Since true mixing becomes progressively poorer in this sequence, errors in the application of the Fick principle of blood flow become greater.

Determination of the pulmonary artery blood flow necessitates measurement of the oxygen content of pulmonary arterial and of pulmonary venous blood. Normally, blood in the main branches of the pulmonary artery is completely mixed. In our limited experience, this is also true in most cases of auricular septal defect. In ventricular septal defect and in patent ductus arteriosus, mixing may be less complete. In these cases, multiple sampling in the pulmonary artery usually yields a representative value from which the calculation of pulmonary blood flow can be made. The oxygen content of pulmonary venous blood is most easily obtained in individuals with a normal circulation and in those with left-to-right shunts by determining the oxygen content of systemic arterial blood since these two are identical. In individuals with right-to-left shunts (cyanotic group), the oxygen content of pulmonary venous blood can be obtained by measuring the oxygen content of pulmonary "capillary" blood (7, 10). Failing this, a value of 95 to 98 per cent saturation must be assumed. This assumption is admittedly open to criticism, especially when pulmonary disease with imperfect oxygen diffusion exists.

The catheter technique does not afford information relative to the collateral circulation between systemic arteries and the pulmonary artery such as sometimes occurs in patients, especially with pulmonary stenosis, if these collateral channels deliver blood to the pulmonary artery beyond the point where catheter samples are obtained. Bing (17) has utilized a combination of the catheter technique and respiratory methods for calculating the magnitude of blood flow through the collateral circulation in these individuals.

The volume of flow through single, one-directional shunts may most easily be estimated by calculating the difference between the pulmonary and peripheral flows. If the shunt is in both directions, as not infrequently occurs, the following formulae,[3] derived by Dr. S. Howard Armstrong, Jr., may be used:

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[3] Let pulmonary artery inflow = a ml. per min. (Determined by Fick equation from pulmonary artery and left auricle oxygen saturations and oxygen consumption).
Let oxygen saturation vena cava inflow $= b$ per cent.
Let oxygen saturation pulmonary vein outflow $= c$ per cent.
Let oxygen saturation pulmonary artery inflow $= d$ per cent.
Now pulmonary artery inflow has 2 components:
(1) from vena cava, x ml. per min.,
(2) from shunt, left-to-right, y ml. per min.
Therefore

$$x + y = a, \tag{1}$$

$$\frac{bx + cy}{x + y} = d. \tag{2}$$

Solving for y, from (2)

$$(b - d)x + (c - d)y = 0$$

$$x = \frac{(d - c)y}{(b - d)}$$

and from (1)

$$y = \frac{a}{1 + \frac{(d - c)}{(b - d)}} = a\,\frac{(b - d)}{(b - c)} \text{ ml. per min.} \tag{3}$$

Now if this is the amount of pulmonary vein blood shunting from left to right and out the pulmonary artery, the remainder must go out the aorta. The other component of aortic flow will shunt from right to left and have the same saturation as right auricular blood, and must equal in amount the return from the vena cava *less* that which has gone through the pulmonary artery. The total caval return and aortic outflow will be assumed to be equal during measurements. Therefore, for total aortic output, u (in ml. per min.), the 2 components are:
(1) from shunt right to left, v ml. per min.,
(2) from the pulmonary veins,

$$e = a\left(1 - \frac{b - d}{b - c}\right) = a\left(\frac{d - c}{b - c}\right).$$

Therefore, $u = v + e$. (4)
Now let f per cent be the oxygen saturation of the peripheral arterial blood. Then

$$f = \frac{bv + ce}{v + e}. \tag{5}$$

Solving (4) for v,

$$(f - b)v = (c - f)e$$

$$v = \frac{(c - f)}{(f - b)}\,e. \tag{6}$$

Where v = right-to-left shunt (l. per min.), y = left-to-right shunt (l. per min.), a = pulmonary blood flow (l. per min.), b = oxygen content (ml. per l.) of mixed venous blood proximal to the shunt (venae cavae, right auricle, or right ventricle), c = oxygen content (ml. per l.) of pulmonary venous blood, d = oxygen content (ml. per l.) of pulmonary arterial blood, and f = oxygen content (ml. per l.) of systemic arterial blood, then

$$y = a\frac{(b-d)}{(b-c)} \qquad v = a\frac{(c-f)(d-c)}{(f-b)(b-c)}.$$

When 2 shunts in the same direction are present in the same heart (*e.g.*, auricular and ventricular septal defect with left-to-right shunts), calculation of the approximate sum total of the shunts is possible, whereas it is not feasible to attempt much more than a guess at the amount of each shunt due to the impossibility of obtaining mixing between the chambers.

All of these difficulties indicate that the Fick principle, when applied to congenital heart disease, gives an estimation of blood flow which is at best an approximation. It is useful to make the calculation, but the assumptions on which such calculations are based must be clearly borne in mind.

Blood pressures: Mean pressures are easily recorded through the venous catheter by means of a simple saline manometer of the venous pressure type. Considerably more information may be obtained with the optical manometer of Hamilton (11). We have experienced difficulty in obtaining suitable pulse wave tracings from the pulmonary artery and from random positions in the right ventricle. With care, good right ventricular pulse wave tracings can be obtained. Artefacts are often set up, apparently by the motion of the catheter within the heart. The natural frequency of the membrane falls from about 170 vibrations per second when recorded through a needle and lead tubing to about 30 vibrations per second when recorded through the catheter and lead tubing. The systolic and diastolic pressures seem to be accurate in that systolic and diastolic pressures in the right ventricle of dogs have been identical when recorded through the catheter and through a No. 18 needle inserted through the chest wall directly into the right ventricle.

The peak of the pressure curve in both pulmonary artery and right ventricle is taken as the systolic pressure, and the level just before the systolic rise is taken as the diastolic pressure. The right auricular pressure is recorded as mean pressure.

The location of the proper level of zero pressure is still controversial since it is different for right auricle, right ventricle, and pulmonary artery. It likewise varies with systole and diastole. Until a standard reference level has been agreed upon, we have chosen to use the level described by one of us (18), which is 10 cm. anterior to the skin of the back with the subject in the supine position. In addition, the anteroposterior diameter of the chest between the sixth thoracic spine and the sternum at the level of the second rib has been recorded with an obstetrical pelvimeter. The pressures, therefore, may be easily recalculated if it is desired to use other zero points.

It has been customary to obtain pressure tracings in the pulmonary artery, right ventricle, and right auricle. A continuous pressure tracing is usually obtained as the catheter is slowly withdrawn from pulmonary artery to right ventricle and from right ventricle to right auricle. In this way comparable pressures are recorded. If the double-lumen catheter of Cournand, Bloomfield, and Lauson (19) is used, pressures may be obtained from 2 chambers simultaneously.

Complications of venous catheterization: That venous catheterization is a benign procedure even in fairly ill patients has been affirmed repeatedly (20, 21, 22), and our experience confirms this. Two sources of mild discomfort have been observed occasionally during venous catheterization. The first is [illegible]

Thus

$$u = c\left(1 + \frac{(c-f)}{(f-b)}\right) = a\frac{(d-c)(c-b)}{(b-c)(f-b)},$$

an expression completely defined in terms of our original quantities measured.
Simplifying algebraically:

Left-to-right shunt $= a\dfrac{(b-d)}{(b-c)}$.

Note that if $(b-d) = 0$, *i.e.*, mixed venous and pulmonary artery blood have same saturation, this means negligible left-to-right shunt.

Right-to-left shunt $= a\left(\dfrac{c-f}{f-b}\right)\left(\dfrac{d-c}{b-c}\right)$.

Note that if $(c-f) = 0$, *i.e.*, left auricle and artery blood have same saturation, this means negligible right-to-left shunt.

ence has been almost uniformly associated with bodily discomfort but has not derived from apprehension. Every effort is made to prevent discomfort. The site of the incision is injected with novocaine about every 10 minutes, the fluoroscope table is covered with a 2-inch sponge rubber mat, pillows are used judiciously for support of the head and shoulders, and the whole procedure is dispatched as quickly as possible (usually about 45 minutes). When venous spasm occurs, it has rarely been possible to relieve it except by removing the catheter from the vein for several minutes.

Ventricular extrasystoles may at times be a source of discomfort to the patient (9). In 27 of 42 patients, irregularity of the heart was shown to occur during venous catheterization. This was demonstrated by electrocardiogram, by pulse tracing, or by palpation. Only 14 of the 27 patients had symptoms referable to this irregularity, and in only 1 were the symptoms of sufficient severity to interrupt the procedure. Five of 12 patients who had electrocardiograms recorded at frequent intervals developed premature ventricular beats. The ventricular extrasystoles are produced especially when the catheter touches the wall adjacent to the tricuspid valve. They usually disappear when the tip of the catheter is in the ventricle, but at times the irregularity persists, apparently due to movement of the catheter with each heartbeat with consequent stimulation of the region of the tricuspid valve. Two patients developed transient auricular fibrillation which subsided spontaneously in the course of half an hour.

In obtaining pulmonary "capillary" blood, the catheter is introduced into the distal portion of a branch of the pulmonary artery so as to obstruct the vessel. It remains in this position for only a few minutes. In no case has there been any change in pulse and respiratory rates and no patient has had untoward symptoms. In dogs a main lobar branch of the pulmonary artery must be obstructed for approximately 1 hour before x-ray and pathological evidence of congestion or infarction appears (23). The vessels occluded by a No. 9–F catheter are roughly 3 mm. in diameter. Since there is a slow drip of physiological saline through the catheter, it seems doubtful whether infarction would occur for many hours.

Auscultation and phonocardiography have revealed no demonstrable changes in the heart sounds or production of murmurs as the catheter passes through either the tricuspid or pulmonary valve. In one patient with pronounced pulmonary stenosis, a catheter about 3 mm. in diameter was introduced past the valve into the pulmonary artery without symptoms. Following a subsequent operation the patient expired, and at autopsy the pulmonary valve orifice was found to measure only 5 mm. in diameter. Cournand, Bloomfield, and Lauson (19) recorded pressure tracings simultaneously in right auricle and right ventricle and concluded that there was no indication of tricuspid insufficiency as a result of inserting the catheter through the tricuspid valve. It seems doubtful, therefore, that venous catheterization produces a functional stenosis or insufficiency of the tricuspid or pulmonary valve under a rather wide range of pathological conditions.

Pathological examination of the endothelium, valve leaflets, chordae tendinae, and papillary muscles in dogs purposely exposed to traumatic venous catheterization of the right auricle, right ventricle, and pulmonary artery has revealed no recognizable pathological lesions resulting from this procedure (24). No evidence of pulmonary embolism from clot formation around the catheter was observed. Post-mortem examinations have been performed on 10 of our patients on whom venous catheterization had previously been performed. In no case was death directly or indirectly attributable to the procedure. In no instance were there any demonstrable lesions of the veins, heart, pulmonary artery, or lung attributable to the passage of the catheter.

SUMMARY

1. The venous catheter technique of Cournand and Ranges (6) has been applied to the diagnosis and study of the hemodynamics of congenital heart disease.

2. Oxygen consumption is measured, and pressures and blood samples (for the oxygen content) are obtained from various parts of the pulmonary artery, right ventricle, right auricle, and from the vena cava and the femoral artery.

3. Details of the procedure are described, and the Fick principle for the calculation of blood flow in patients with congenital heart disease is discussed.

BIBLIOGRAPHY

1. Gross, R. E., and Hubbard, J. P., Surgical ligation of a patent ductus arteriosus. Report of first successful case. J. A. M. A., 1939, 112, 729.
2. Blalock, A., and Taussig, H. B., Surgical treatment of malformations of the heart. J. A. M. A., 1945, 128, 189.
3. Crafoord, C., and Nylin, G., Congenital coarctation of aorta and its surgical treatment. J. Thoracic Surg., 1945, 14, 347.
4. Gross, R. E., and Hufnagel, C. A., Coarctation of the aorta. Experimental studies regarding its surgical correction. New England J. Med., 1945, 233, 287.
5. Forssman, W., Die Sondierung der rechten Herzens. Klin. Wchnschr., 1929, 8, 2085.
6. Cournand, A., and Ranges, H. A., Catheterization of right auricle. Proc. Soc. Exper. Biol. & Med., 1941, 46, 462.
7. Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Sagerson, R. P., and Evans, J. M., Studies of congenital heart disease. II. The pressure and oxygen content of blood in the right auricle, right ventricle, and pulmonary artery in control patients, with observations on the oxygen saturation and source of pulmonary "capillary" blood. J. Clin. Invest., 1947, 26, 554.
8. Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Sosman, M. C., and Evans, J. M., Studies of congenital heart disease. III. Venous catheterization as a diagnostic aid in patent ductus arteriosus, tetralogy of Fallot, ventricular septal defect, and auricular septal defect. J. Clin. Invest., 1947, 26, 561.
9. Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. deF., and Richards, D. W., Jr., Measurement of cardiac output in man using the technique of catheterization of the right auricle or ventricle. J. Clin. Invest., 1945, 24, 106.
10. Dexter, L., Burwell, C. S., Haynes, F. W., and Seibel, R. E., Oxygen content of pulmonary "capillary" blood in unanesthetized human beings. J. Clin. Invest., 1946, 25, 913.
11. Hamilton, W. F., Brewer, G., and Brotman, I., Pressure pulse contours in the intact animal. I. Analytical description of a high frequency manometer. Am. J. Physiol., 1934, 107, 427.
12. Cournand, A., Personal communication, 1945.
13. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry, Vol. 2, Methods. Williams & Wilkins, Baltimore, 1943.
14. Fick, A., Ueber die Messung des Blutquantums in den Herzventrikeln. Verhandl. d. physical. med. gesellsch. z. Würzburg, 1870, 2, 16.
15. Eppinger, E. C., Burwell, C. S., and Gross, R. E., The effects of the patent ductus arteriosus on the circulation. J. Clin. Invest., 1941, 20, 127.
16. Cournand, A., Measurement of the cardiac output in man using the right heart catheterization. Description of technique, discussion of validity and of place in the study of the circulation. Federation Proc., 1945, 4, 207.
17. Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., Physiological studies in congenital heart disease. I. Procedures. Bull. Johns Hopkins Hosp., 1947, 80, 107.
18. Lyons, R. H., Kennedy, J. A., and Burwell, C. S., Measurement of venous pressure by direct method. Am. Heart J., 1938, 16, 675.
19. Cournand, A., Bloomfield, R. A., and Lauson, H. D., Double lumen catheter for intravenous and intracardiac blood sampling and pressure recording. Proc. Soc. Exper. Biol. & Med., 1945, 60, 73.
20. Cournand, A., Riley, R. L., Bradley, S. E., Breed, E. S., Noble, R. P., Lauson, H. D., Gregersen, M. I., and Richards, D. W., Studies on the circulation in clinical shock. Surgery, 1943, 13, 964.
21. McMichael, J., and Sharpey-Schafer, E. P., Cardiac output in man by direct Fick method; effects of posture, venous pressure change, atropine, and adrenaline. Brit. Heart J., 1944, 6, 33.
22. Brannon, E. S., Weens, H. S., and Warren, J. V., Atrial septal defect. Study of hemodynamics by the technique of right heart catheterization. Am. J. M. Sc., 1945, 210, 480.
23. Kinney, T. D., Haynes, F. W., and Dexter, L., Unpublished observations, 1946.
24. Kinney, T. D., Haynes, F. W., and Dexter, L., Experimental production of pulmonary embolism by the use of a venous catheter. J. Lab. & Clin. Med., 1945, 30, 1013.

STUDIES OF CONGENITAL HEART DISEASE. II. THE PRESSURE AND OXYGEN CONTENT OF BLOOD IN THE RIGHT AURICLE, RIGHT VENTRICLE, AND PULMONARY ARTERY IN CONTROL PATIENTS, WITH OBSERVATIONS ON THE OXYGEN SATURATION AND SOURCE OF PULMONARY "CAPILLARY" BLOOD[1]

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The venous catheter of Cournand and Ranges (1) permits the measurement of pressure and oxygen content of blood in the pulmonary artery, right ventricle, and right auricle of patients with congenital heart disease. To interpret the data so obtained, it is necessary to be cognizant of the variations in these measurements that occur in patients without congenital cardiac defects. The present report deals with the findings in a group of control patients.

METHODS AND MATERIAL

The procedures of measuring oxygen consumption, of blood sampling, and of pressure recording in various parts of the right side of the heart have been described in detail in a previous paper (2). The oxygen content of blood was determined by the method of Van Slyke and Neill (3), and pressures were recorded by the optical manometer of Hamilton (4). As noted previously, the zero point for all pressures was taken as 10 cm. anterior to the skin of the back with the patient in the supine position (5). In addition, the anteroposterior diameter of the chest between the sixth thoracic vertebra and the angle of Louis has been recorded for the benefit of those wishing to use another point of reference.

In 13 patients without any evidence of congenital heart disease, the oxygen content of blood and pressures in various parts of the pulmonary artery, right ventricle, and right auricle have been studied (see Table I).

Fifteen patients with a variety of disorders but without clinical or laboratory evidence of auricular septal defect, as noted in Table II, were studied for the purpose of comparing the oxygen content of blood in the right auricle with that in the superior and inferior venae cavae.

In a third group of 16 patients with a variety of abnormalities but without cyanosis (see Table III), the oxygen content of blood obtained from the pulmonary "capillaries" was determined. In addition, 3 patients with right-to-left shunts have been included (see Table IV). Two of these patients had auricular septal defects (confirmed by autopsy in one case), and the third had the tetralogy of Fallot.

By consulting Tables I, II, and III, it will be noted that the majority of patients were not normal. They were chosen on 2 bases: that they had no defect which would interfere with the interpretation of the results and that they did have a number of abnormalities, such as cardiac failure, anemia, and pulmonary disease, which are sometimes associated with congenital heart lesions. It is believed, therefore, that the values obtained in these patients serve as useful controls for the values observed in patients with congenital cardiac defects.

OBSERVATIONS AND INTERPRETATIONS

Pressures: Figure 1 illustrates pressure tracings from the pulmonary artery, right ventricle, and right auricle in a normal individual. Gross differences in the pulse wave forms in the different chambers are apparent.

The recording of pressures in patients with congenital heart disease is of particular value in the detection of pulmonary stenosis (6). The systolic pressures in the pulmonary artery and right ventricle were identical within the limit of respiratory variations in each of the patients in Table I. This is in contrast to the findings in pulmonary stenosis where the pulse pressure in the pulmonary artery tends to be narrow and the systolic pressure in the right ventricle is clearly higher than that in the pulmonary artery (6). It is believed that the systolic and diastolic pressures recorded through the catheter with the Hamilton manometer are accurate (2). The finer structure of the pulse waves, especially in the pulmonary artery, is so subject to artefacts that it has not been used by us for analytical purposes. Nevertheless, differences in the gross configura-

[1] This investigation was aided in part by a grant from the John and Mary Markle Foundation, and from the Proctor Fund of the Harvard Medical School.

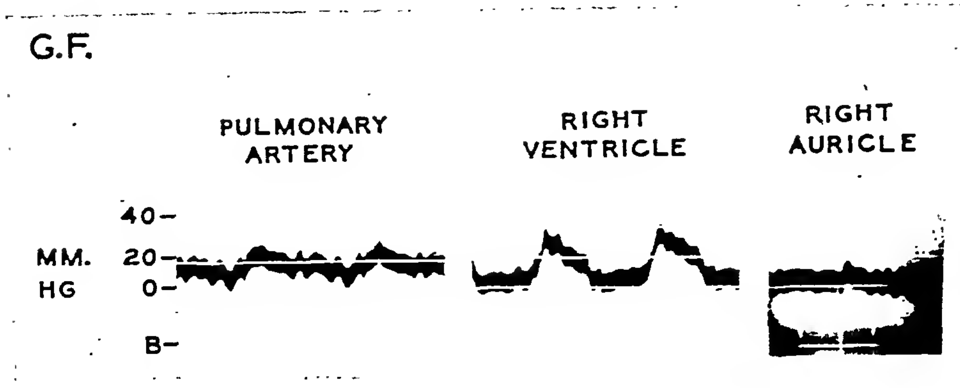

FIG. 1. PRESSURE TRACINGS IN PULMONARY ARTERY, RIGHT VENTRICLE, AND RIGHT AURICLE OF A NORMAL PATIENT

The systolic pressures in the pulmonary artery and right ventricle are almost identical, the differences observed being attributable to respiratory fluctuations. Artefacts in the tracings are probably due to the motion of the catheter within the heart and pulmonary artery.

tion of these waves are of the utmost value in identifying the chamber in which the tip of the catheter is located, especially when it lies close to either the pulmonary or the tricuspid valve. This can easily be accomplished at the time of catheterization by noting the deflection of the light beam as the catheter is withdrawn from one chamber to another.

Variations of oxygen content between different chambers: In congenital heart disease, left-to-right shunts introduce arterial blood into the right auricle, right ventricle, or pulmonary artery, thereby increasing the oxygen content of blood within the chamber. Recognition of a significant increase of oxygen in the blood of a given chamber requires a knowledge of the variations that may occur normally. In our control patients (Tables I and II), the greatest increase in the oxygen content of blood in the right auricle over that present in the superior vena cava was 1.9 volumes per cent (Table II, patient 9). The greatest increase in the oxygen content of blood in the right ventricle over that present in any part of the right auricle was 0.9 volume per cent (Table I, patients 5 and 12). The greatest increase in the oxygen content of blood in the pulmonary artery over that present in any part of the right ventricle was 0.5 volume per cent (Table I, patient 4). These values are at the moment considered as maximal normal variations with the tentative postulation that a wider divergence is indicative of a shunt of arterial blood into the given chamber. With a larger series of control patients, it is possible that variations encountered between the various chambers would be greater.

It is obvious that left-to-right shunts of small size may be easily overlooked unless the operator can place the tip of the catheter in the stream of arterial blood flowing through the abnormal communication rather than in areas where the arterial stream has become mixed with the surrounding venous blood. This requires knowledge of the probable location of such a shunt as regards not only the chamber involved but also the location of the defect within that chamber. Careful evaluation of the patient by other diagnostic methods is, therefore, essential before performing venous catheterization.

Attempts were made to obtain representative samples of blood from the inferior vena cava by withdrawing the catheter from the region of the renal vein to a point a few centimeters below the diaphragmatic shadow and withdrawing blood samples without further manipulation of the catheter. The oxygen content of blood from the inferior vena cava was uniformly higher than the maximal value obtained from the right auricle. This may well have been due to withdrawal of blood coming predominantly from the renal vein which contains a high content of oxygen (7). In view of this uniform finding, we have [illegible] the sampling of blood from the inferior vena cava [illegible] for comparative purposes [illegible] from the superior vena cava.

TABLE I

Oxygen content of blood and pressures in pulmonary artery, right ventricle, and right auricle of control group of patients

Patient no.	Initials	Diagnosis	Age	Oxygen content: Pulmonary artery: Main branch	Pulmonary artery: Main stem	Pulmonary artery: Variation	Right ventricle: Near pulmonary valve	Right ventricle: Mid-portion	Right ventricle: Near tricuspid valve	Right ventricle: Variation	Right auricle: Near tricuspid valve	Right auricle: Lower portion	Right auricle: Upper portion	Right auricle: Variation	Variations of maximal values: Pulmonary artery and right ventricle	Variations of maximal values: Right ventricle and right auricle	Pressure: Pulmonary artery	Pressure: Right ventricle	Pressure: Right auricle	A-P diameter of chest
				vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	mm. Hg	mm. Hg	mm. Hg	cm.
1	B. P.	No disease	15	14.4	14.6	0.2	14.5	14.4	14.8	0.4	14.5		14.4	0.1	−0.2	+0.3	32/12	32/6	6	15.5
2	G. F.	No disease	17	15.6	15.3	0.3	14.6	14.8	15.4	0.8	15.2		15.3	0.1	+0.2	+0.1	20/8	20/0	0	16.0
3	S. I.	Patent ductus arteriosus divided 17 days before	7	13.6	13.7	0.1	13.5	13.3	13.6	0.3	13.4		13.4	0.0	+0.1	+0.2	30/10	30/0	0	14.5
4	F. R.	Pneumonia convalescent; chronic alcoholism	33	13.2	13.3	0.1	12.8		12.8	0.0	12.2	12.1	12.0	0.2	+0.5	+0.6	26/10	26/5	5	
5	N. F.	Unresolved pneumonia	21	14.1			14.0		13.4	0.6		12.9	13.1	0.2	+0.1	+0.9	30/10	30/5	5	21.0
6	J. H.	Essential hypertension	42	13.7	13.3	0.4	13.7		13.7	0.0		14.5	13.4	1.1	0.0	−0.8	33/13	33/7	7	21.0
7	R. D.	Acute rheumatic fever	13	11.5	11.5	0.0	11.9	11.6	11.7	0.3	11.5		11.4	0.1	−0.4	−0.4	20/8	20/4	4	17.0
8	R. A.	Treated thyrotoxicosis; B.M.R. + 11	21	15.4	15.5	0.1	15.7		15.7	0.0	15.4	15.8	15.7	0.4	−0.2	−0.1	30/12	30/8	8	19.0
9	G. O.	Kyphoscoliosis; ? cardiac failure	43	13.5	13.3	0.2	13.3		12.8	0.5	13.2		13.3	0.1	+0.2	0.0	58/36	59/17	17	30.0
10	F. B.	Emphysema; ? cardiac failure	57	13.9	13.9	0.0	13.1	13.5		0.4			13.5		+0.4	0.0	55/43	55/28	28	30.0
11	C. C.	Cardiac failure; ? cause	54	10.7	10.7	0.0	10.7	10.8	10.7	0.1	10.6		10.7	0.1	−0.1	+0.1	60/18	60/12	12	23.0
12	M. Q.	Pulmonary vascular disease	32	13.7	13.5	0.2	13.7		13.1	0.6	12.7	12.8	12.7	0.1	0.0	+0.9	*80/55	80/20	8	18.0
13	I. G.	Patent ductus arteriosus divided 14 days before; anemia; cardiac failure	26	9.2	9.1	0.1	9.2	8.8	8.8	0.4	7.5		8.8	1.3	0.0	+0.4	80/45	60/8 80/−3	−3	17.0

* Upper readings obtained withdrawing catheter from pulmonary artery to right ventricle; lower, from right ventricle to right auricle.

TABLE II

Variations of oxygen content of blood withdrawn from each vena cava and the right auricle

Patient no.	Initials	Diagnosis	Oxygen content						Difference between highest value in right auricle and that in the**	
			Superior vena cava	Right auricle				Inferior vena cava	Superior vena cava	Inferior vena cava
				Upper portion	Near tricuspid valve	Right middle portion	Lower portion			
			vol. per cent	*vol. per cent*				*vol. per cent*		
1	G. F.	Normal	14.8	*15.3**		15.2			−0.5	
2	S. I.	Patent ductus arteriosus	9.8	10.0		*10.4*			−0.6	
3	R. D.	Rheumatic fever, convalescent	10.3		*11.5*	11.4			−1.2	
4	H. G.	Tetralogy of Fallot	17.6	*18.6*	7.2	18.5			−1.0	
5	J. D.	Normal	15.7	*16.0*	10.5			18.0	−0.3	+2.0
6	J. H.	Aortic insufficiency, ventricular septal defect	12.8	12.1	*12.6*				+0.2	
7	H. R.	Duodenal ulcer	13.1	13.4	*13.6*			15.1	−0.5	+1.5
8	L. S.	Bronchial asthma	14.3	15.1	14.1		*15.6*	16.5	−1.3	+0.9
9	M. T.	Lues, treated	9.5	10.9	*11.4*		11.0	12.9	−1.9	+1.5
10	S. O.	Cystitis; pneumonia, convalescent	8.7	9.3	10.0		*10.1*	10.3	−1.4	+0.2
11	G. B.	Rheumatic heart disease	10.2	10.1		*10.4*	*10.4*		−0.2	
12	J. M.	Uncomplicated pulmonary stenosis	14.4	14.3	*15.2*		*15.2*		−0.8	
13	M. R.	Chorea, mitral stenosis, aortic insufficiency	14.3	15.2		*15.4*			−1.1	
14	G. J.	Peptic ulcer	10.6	10.9	11.1		*11.5*	12.1	−0.6	+0.6
15	H. G.	Spontaneous pneumothorax	16.4	*17.0*		16.4		17.4	−0.6	+0.4

* Figures in italics represent the highest right auricular values.
** (+) denotes a higher value in the right auricle than in the vena cava; (−) denotes a lower value.

Variation of oxygen content within each chamber: The maximal variation of oxygen content of blood samples obtained from different parts of the right auricle was 11.4 volumes per cent (Table II, patient 4), and 5.5 volumes per cent (Table II, patient 5). In each instance, one sample was highly unoxygenated. The maximal variation within the right ventricle was 0.8 volume per cent (Table I, patient 2). Greater variations than these have been observed in the right ventricle of patients not included in Table I. In 2 patients, variations of 4.5 and 8.4 volumes per cent have been recorded, one of the samples in each case being highly unsaturated. The maximal variation between the trunk and one of the main branches of the pulmonary artery was only 0.4 volume per cent (Table I, patient 6) and 0.3 volume per cent (Table I, patient 2).

The accuracy of the direct Fick method for the determination of cardiac output under a given set of conditions depends on the accuracy with which the oxygen consumption and the oxygen content of arterial and mixed venous bloods are determined. This problem has been discussed critically by Cournand (8), who pointed out that whereas the first two factors are determinable with reasonable accuracy, the third (mixed venous blood) may occasionally be difficult to obtain from the right auricle. Holt and Knoefel (9) reported that true mixing did not occur in the right auricle of dogs. In man, however, Cournand and his associates (8, 10, 11) concluded that, if the tip of the catheter is located with care in the right auricle close to the tricuspid valve, mixed venous blood is usually obtainable. They showed, however, that even with the catheter in this position in the right auricle, the samples withdrawn were sometimes poorly mixed, as in our case 13 of Table I and in cases 4 and 5 of Table II. Warren, Stead, and Brannon (12) found a maximum variation of oxygen content in the auricle of 2.3 volumes per cent, and in the ventricle 1.8 volumes per cent. They concluded that neither the auricle nor the ventricle could be considered [illegible]

venous blood in individual cases, although if groups of cases were treated statistically, good mixing could be considered to have taken place.

Our data confirm the findings of Cournand and his associates and of Warren, Stead, and Brannon in indicating that although adequate mixing usually occurs in the right auricle and right ventricle, unreliable values may at times be obtained. The abnormal values are almost uniformly attributable to low oxygen content of one sample, presumably due to the entrance of coronary venous blood with its low oxygen content (13) into the right ventricle by way of Thebesian vessels and into the right auricle by way of the coronary sinus. It is possible that hepatic venous blood which is low in oxygen (14) may be responsible for the low values in some of the samples obtained from the right auricle.

In contrast to the occasional discrepancies in the oxygen content of blood obtained from right auricle and right ventricle, there was remarkable uniformity in the values obtained from the pulmonary artery. The maximal variations observed hardly exceeded the error of the method of determining oxygen content. It is believed, therefore, that true mixing of venous blood occurs uniformly in the pulmonary artery in man, and that the use of the pulmonary artery as a source of mixed venous blood in determining cardiac outputs by the direct Fick principle will overcome one of the major sources of error of this technique.

Pulmonary "capillary" blood: The oxygen content and saturation of blood obtained through the catheter, when it is introduced into a distal branch of the pulmonary artery so as to occlude it, are shown in Table III. It will be observed that in all cases there was close agreement between the oxygen saturation of this blood and that taken from the femoral artery. In no case did the oxygen saturation of blood from the pulmonary "capillaries" exceed that from the femoral artery by more than 2 per cent. Samples taken from other locations in the pulmonary artery uniformly had lower oxygen saturations. This was also true in the 3 patients with patent ductus arteriosus.

The arterial blood withdrawn when the pulmonary artery is obstructed by the catheter might be derived from anastomoses with bronchial arteries or from the pulmonary capillary and venous bed. The existence of bronchial artery anastomoses proximal to the pulmonary capillaries under normal conditions is doubtful (15, 16), although un-

TABLE III

Oxygen content, capacity, and saturation of blood from femoral artery, pulmonary "capillaries," and pulmonary artery in patients without cyanosis

Patient no.	Initials	Diagnosis	Femoral artery			Pulmonary "capillaries"			Pulmonary artery (highest value)		
			Oxygen content	Oxygen capacity	Oxygen saturation	Oxygen content	Oxygen capacity	Oxygen saturation	Oxygen content	Oxygen capacity	Oxygen saturation
			vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent	per cent	vol. per cent	vol. per cent	per cent
1	F. D.	Convalescent beriberi	17.2	18.2	94	16.9	(18.2)*	93	13.4	(18.2)	74
2	A. L.	Patent ductus arteriosus	16.2	16.8	97	16.5	(16.8)	98	15.5	(16.8)	92
3	E. R.	Pulmonary stenosis	18.4	18.5	100	17.5	17.7	99	15.2	(17.7)	86
4	J. H.	Ventricular septal defect	17.7	17.7	100	17.6	17.7	100	16.0	(17.7)	91
5	L. C.	Ventricular septal defect	14.5	14.7	99	14.2	14.7	97	11.7	(14.7)	80
6	T. B.	Patent ductus arteriosus	17.2	18.2	94	17.3	18.0	96	15.3	(18.1)	85
7	R. A.	Thyrotoxicosis, treated				18.6	19.3	96	15.5	(19.3)	80
8	M. C.	Epididymitis				18.4	18.9	97	14.8	(18.9)	78
9	S. I.	Patent ductus arteriosus				15.6	16.1	97	13.7	16.1	85
10	R. D.	Acute rheumatic fever				15.8	15.5	100	11.5	15.5	75
11	C. C.	Cardiac failure, ? cause	16.6	16.7	100	16.2	16.7	98	10.7	16.7	64
12	G. F.	Normal	18.4	18.5	99	18.2	18.5	98	15.6	18.5	84
13	B. P.	Normal	19.0	19.6	97	14.7	14.9	99	14.6	19.6	75
14	R. J.	Auricular and ventricular septal defects	18.2	18.6	98	18.0	18.1	99	17.2	18.6	92
15	J. D.	? congenital dilatation of pulmonary artery	21.3	22.4	95	20.9	21.7	97	17.3	22.4	77
16	I. G.	Patent ductus arteriosus	16.7	17.3	97	14.6	15.1	97	15.1	16.4	93

* Parentheses indicate that neither the oxygen capacity nor the hemoglobin was determined on the individual sample.

TABLE IV

Oxygen content, capacity, and saturation of blood from femoral artery, pulmonary "capillaries," and pulmonary artery in patients with right-to-left shunts

Patient no.	Initials	Diagnosis	Femoral artery: Oxygen content	Femoral artery: Oxygen capacity	Femoral artery: Oxygen saturation	Pulmonary "capillaries": Oxygen content	Pulmonary "capillaries": Oxygen capacity	Pulmonary "capillaries": Oxygen saturation	Pulmonary artery (highest value): Oxygen content	Pulmonary artery (highest value): Oxygen capacity	Pulmonary artery (highest value): Oxygen saturation	How diagnosed
			vol. per cent	*vol. per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*per cent*	
1	J. S.	Auricular septal defect, chronic nephritis, hypertension, anemia	8.1	8.7	94	8.7	(8.8)*	99	7.5	(8.8)	86	Autopsy
2	A. C.	Auricular septal defect, secondary polycythemia	25.0	32.5	76	18.5	20.5	90	15.8	25.6	62	Venous catheterization
3	E. E.	Ventricular septal defect, and pulmonary stenosis	21.1	24.2	87	22.7	24.2	94	17.8	24.2	74	Venous catheterization

* Parentheses indicate that neither the oxygen capacity nor the hemoglobin was determined on the individual sample.

der certain circumstances anastomoses between large bronchial arteries and pulmonary arteries may be striking (17). The finding of a significantly higher oxygen saturation of blood withdrawn from the distal part of the pulmonary artery than in that from the femoral artery in 3 patients with cyanosis from a right-to-left shunt (Table IV) indicates that the blood so obtained does not derive from direct anastomoses with the systemic arterial circulation. It seems justifiable to conclude that it is aspirated mainly from the pulmonary capillary and venous bed. A small pre-capillary source cannot be excluded by these data.

The finding of an oxygen saturation of 95 to 100 per cent in the pulmonary "capillary" blood excludes the existence of pulmonary disease with oxygen diffusion difficulties. The finding of a diminished oxygen saturation, however, does not indicate the presence of pulmonary disease since there may well have been only partial obstruction of the pulmonary arterial branch with some admixture of venous blood from the pulmonary artery. If, in cyanotic patients, values for oxygen saturation in femoral artery and pulmonary "capillaries" are found to be identical and reduced, it is presumptive evidence that the oxygen unsaturation is on the basis of disordered pulmonary function.

Diminution of the oxygen capacity of some of the samples obtained from the pulmonary "capillaries" (see Table II) may well be related to the decreased hematocrit of capillary blood which has been found in samples taken elsewhere in the body (18). Studies of the cause of the variations observed are in progress.

SUMMARY

1. Venous catheterization of the venae cavae, right auricle, right ventricle, and pulmonary artery has been performed in control patients with various diseases but without congenital defects affecting the factors studied.

2. Pressures and multiple samples of blood for oxygen analysis have been obtained from each chamber.

3. The greatest increase in oxygen from the superior vena cava to the right auricle was 1.9 volumes per cent; from the right auricle to the right ventricle, 0.9 volume per cent; and from the right ventricle to the pulmonary artery, 0.5 volume per cent.

4. The systolic pressure in the pulmonary artery was almost identical with that in the right ventricle over a wide range of pressures.

5. Arterial blood may be obtained from the distal branches of the pulmonary artery by obstructing the artery with the catheter and withdrawing blood through the catheter lumen. Evidence is presented indicating that this blood originates in the pulmonary capillary and venous bed rather than in pre-capillary anastomoses with systemic arteries.

6. The oxygen content of bloods obtained from several sites within the right auricle and right ventricle showed at times considerable variation, while those from the pulmonary artery were remarkably uniform. It is concluded that mixed venous blood for the determination of cardiac output may be obtained consistently from the pulmonary artery but not uniformly from the right auricle and right ventricle.

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BIBLIOGRAPHY

1. Cournand, A., and Ranges, H. A., Catheterization of the right auricle. Proc. Soc. Exper. Biol. & Med., 1941, 46, 462.
2. Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Seibel, R. E., and Evans, J. M., Studies of congenital heart disease. I. Technique of venous catheterization as a diagnostic procedure. J. Clin. Invest., 1947, 26,
3. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Vol. 2, Methods. Williams & Wilkins, Baltimore, 1943.
4. Hamilton, W. F., Brewer, G., and Brotman, I., Pressure pulse contours in the intact animal. I. Analytical description of a high frequency manometer. Am. J. Physiol., 1934, 107, 427.
5. Lyons, R. H., Kennedy, J. A., and Burwell, C. S., Measurement of venous pressure by direct method. Am. Heart J., 1938, 16, 675.
6. Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Sosman, M. C., and Evans, J. M., Studies of congenital heart disease. III. Venous catheterization as a diagnostic aid in patent ductus arteriosus, tetralogy of Fallot, ventricular septal defect, and auricular septal defect. J. Clin. Invest., 1947, 26, 561.
7. Warren, J. V., Brannon, E. S., and Merrill, A. J., A method of obtaining renal venous blood in unanesthetized persons with observations on the extraction of oxygen and sodium para-amino hippurate. Science, 1944, 100, 108.
8. Cournand, A., Measurement of the cardiac output in man using the right heart catheterization. Description of technique, discussion of validity and of place in the study of the circulation. Federation Proc., 1945, 4, 207.
9. Holt, J. P., and Knoefel, P. K., Oxygen content of blood in right auricle and right ventricle. Federation Proc., 1944, 3, 19.
10. Cournand, A., Bloomfield, R. A., and Lauson, H. D., Double lumen catheter for intravenous and intracardiac blood sampling and pressure tracing. Proc. Soc. Exper. Biol. & Med., 1945, 60, 73.
11. Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. deF., and Richards, D. W., Jr., Measurement of cardiac output in man using the technique of catheterization of the right auricle or ventricle. J. Clin. Invest., 1945, 24, 106.
12. Warren, J. V., Stead, E. A., Jr., and Brannon, E. S., Cardiac output in man: A study of some of the errors in the method of right heart catheterization. Am. J. Physiol., 1946, 145, 458.
13. Harrison, T. R., Failure of the Circulation. Williams & Wilkins, Baltimore, 1935, p. 248.
14. Warren, J. V., and Brannon, E. S., A method of obtaining blood samples directly from the hepatic vein in man. Proc. Soc. Exper. Biol. & Med., 1944, 55, 144.
15. Ghoreyeb, A. A., and Karsner, H. T., A study of the relation of pulmonary and bronchial circulation. J. Exper. Med., 1913, 18, 500.
16. Miller, W. S., The Lung. Charles C. Thomas, Springfield, 1937.
17. Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., Physiological studies in congenital heart disease. II. Results of preoperative studies in patients with tetralogy of Fallot. Bull. Johns Hopkins Hosp., 1947, 80, 121.
18. Ebert, R. V., and Stead, E. A., Jr., Demonstration that the cell plasma ratio of blood contained in the minute vessels is lower than that of venous blood. J. Clin. Invest., 1941, 20, 317.

STUDIES OF CONGENITAL HEART DISEASE. III. VENOUS CATHETERIZATION AS A DIAGNOSTIC AID IN PATENT DUCTUS ARTERIOSUS, TETRALOGY OF FALLOT, VENTRICULAR SEPTAL DEFECT, AND AURICULAR SEPTAL DEFECT[1]

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Previous communications have outlined the technique of cardiac catheterization as applied to the study of congenital heart disease and defined the variations in pressures and oxygen content observed in a group of control patients (1, 2). This paper sets forth the results of study in 6 patients with 4 varieties of congenital heart disease as examples of the type of information which can be acquired by the catheter technique in such patients.

OBSERVATIONS

Patent ductus arteriosus

Patent ductus arteriosus is a persistence after birth of the vascular anastomosis between the pulmonary artery and the aorta. While in the fetus blood flows from the pulmonary artery to the aorta, the flow is from the aorta to the pulmonary artery after birth, as Eppinger, Burwell, and Gross (3) have demonstrated.

Case 1. I. G. was a 26-year-old woman. In early infancy she was found to have a heart murmur. She developed normally but was subject to palpitation, dyspnea, and fatigue on exertion. On physical examination, she was a well-developed woman without cyanosis or clubbing. Arterial blood pressure was 125/65 mm. Hg. The heart was enlarged to the left. There was a machinery murmur in the pulmonic region. The first sound at the apex and the second sound at the pulmonic area were accentuated. The lungs were clear, the liver was not enlarged, and no edema was apparent. X-ray of the heart (Figure 1) showed it to be about 20 per cent enlarged with prominence in the region of the pulmonary artery and with hilar engorgement. An electrocardiogram was normal.

Venous catheterization was performed, and Table I records the pressures and oxygen contents of blood samples obtained from the various points shown in Figure 1. It will be noted that the oxygen content of blood in the pulmonary artery was 2.2 volumes per cent higher than of that from the right ventricle. This is taken as indicating the entrance of arterial blood into the pulmonary artery. Arterial shunts, other than patent ductus arteriosus, are exceedingly rare in this region. The systolic pressures in the pulmonary artery and right ventricle were elevated. The shunt was calculated to be 7.6 l. per min.

A few days later, Dr. Robert E. Gross ligated and divided a patent ductus arteriosus. Two weeks later, venous catheterization was repeated, at which time the patient was weak and moderately anemic (hemoglobin 9.9 grams). Results

TABLE I

I. G. Age 26. Patent ductus arteriosus

Sample	Oxygen content	Pressure
	vol. per cent	*mm. Hg*
Left pulmonary artery (near bifurcation)	15.1	63/35
Right pulmonary artery (near bifurcation)	15.1	
Right ventricle (near pulmonary valve)	12.9	63/6
Right ventricle (mid-portion)	12.5	
Right auricle (near tricuspid valve)	13.0	6
Right auricle (upper portion)	11.9	
Systemic artery	16.7 (97 per cent)	125/65

[1] This investigation was aided in part by a grant from the John and Mary R. Markle Foundation, and from the Proctor Fund of the Harvard Medical School.

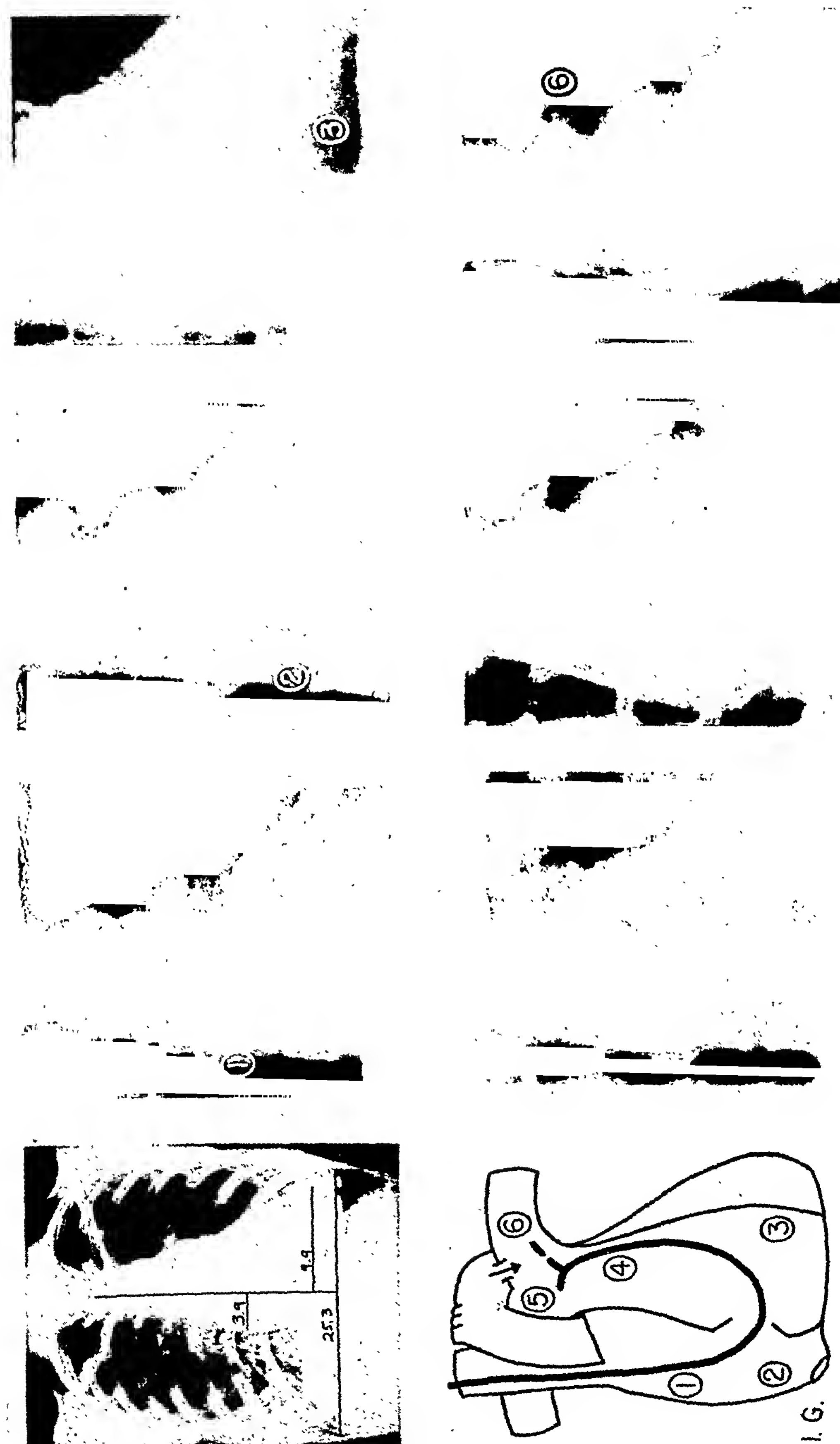


FIG. 1. PATENT DUCTUS ARTERIOSUS (CASE 1)
The positions of the catheter are identifiable by the corresponding numbers in the schema.

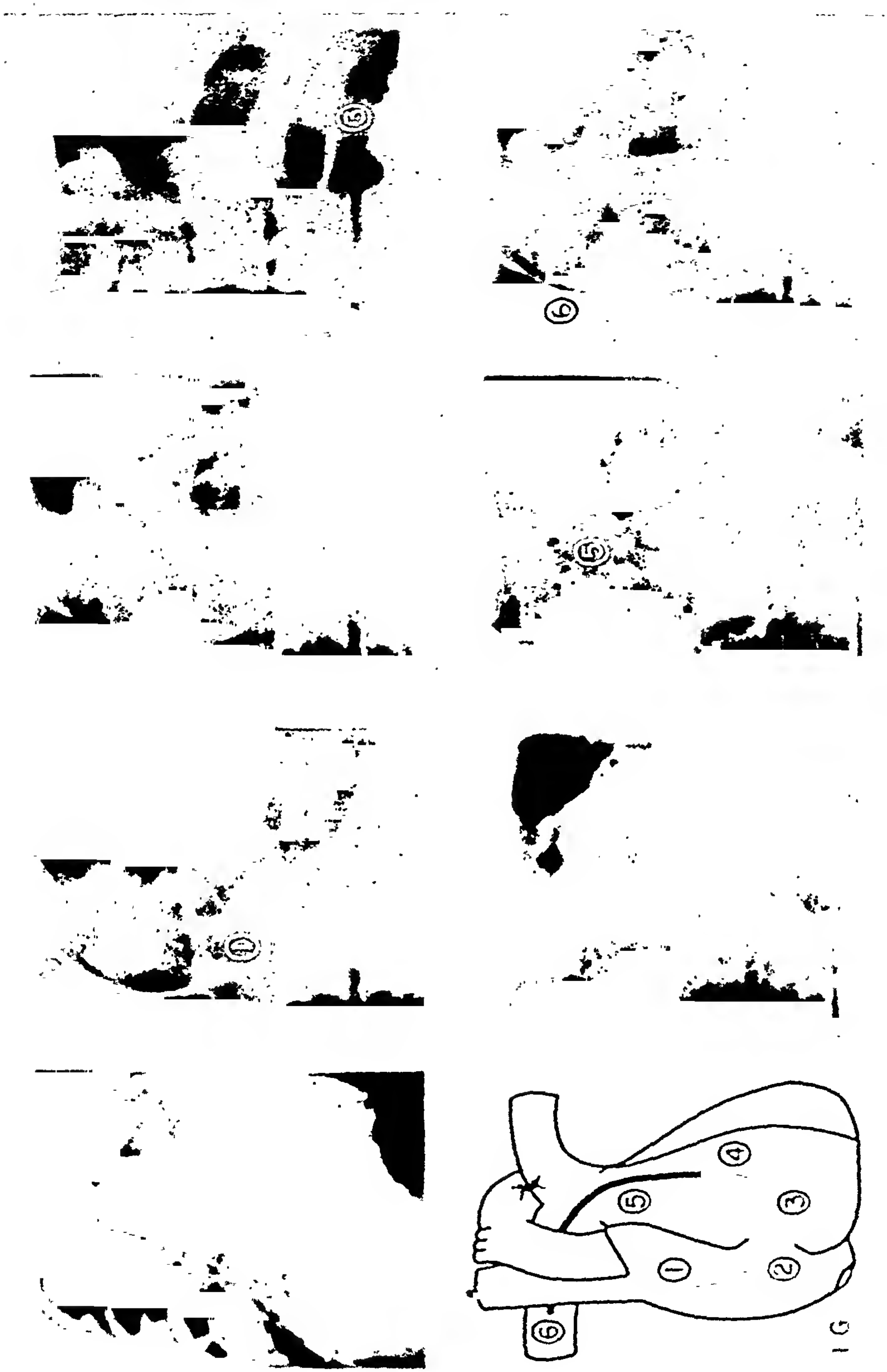


FIG. 2. TWO WEEKS AFTER DIVISION OF THE PATENT DUCTUS ARTERIOSUS (CASE 1)
The positions of the catheter are identifiable by the corresponding numbers in the schema.

of the catheterization are presented in Figure 2 and Table II. The oxygen values of bloods from the 3 chambers were in good agreement and showed no deviation from the normal. The pressures in the pulmonary artery and right ventricle were still elevated.

TABLE II

I. G. Age 26. 14 days after division of patent ductus arteriosus

Sample	Oxygen content	Pressure
	ml. per l.	*mm. Hg*
Right pulmonary artery (mid-portion)	91	80/45
Right pulmonary artery (near bifurcation)	92	
Right ventricle (near pulmonary valve)	92	80/−3
Right ventricle (mid-portion)	88	
Right ventricle (near tricuspid valve)	88	
Right auricle (near tricuspid valve)	75	−3
Right auricle (upper portion)	88	
Systemic artery	124 (90 per cent)	136/88

A–P diameter of chest	17 cm.
Oxygen consumption	244 ml. per min.
Body surface area	1.50 sq. m.
Arteriovenous oxygen difference	32 ml. per l.
Cardiac output	7.6 l. per min.
Cardiac index	5.1

Discussion: Calculation of the volume of flow through the ductus was made first by Eppinger, Burwell, and Gross (3) on the basis of blood samples obtained from the pulmonary artery at the time of operation. Blood samples withdrawn through the intracardiac catheter have the great advantage of being obtained with the patient in a relatively basal state. An oxygen content of blood in the pulmonary artery significantly higher than that in the right ventricle has been found regularly by venous catheterization in patients with patent ductus arteriosus.

In a previous study with the catheter in a control series of patients (2), it was observed that the greatest variation between the highest oxygen content of blood in the right ventricle and that in the pulmonary artery was 0.5 volume per cent. On this basis, it is believed that flows through the ductus of less than 0.5 l. per min., and possibly as high as 1.0 l. per min., are probably not detectable by the venous catheter method unless the tip of the catheter can be placed in the stream of arterial blood flowing into the pulmonary artery through the ductus, in which case smaller defects may be detectable.

Some patients with patent ductus have shown an elevation of pressure in the pulmonary artery and right ventricle, as in the example cited. In the majority of patients, however, the pressures have been normal.

The diagnosis of patent ductus arteriosus has been confirmed by venous catheterization in 7 patients to date and has been proved subsequently at the time of operation in each.

Tetralogy of Fallot

The tetralogy of Fallot consists of pulmonary stenosis, interventricular septal defect, dextroposition of the aorta, and right ventricular hypertrophy. There is a diminished blood flow to the lung. A shunting of blood from the right ventricle into the aorta produces cyanosis.

Case 2. P. B. was 22 years old and had been a "blue baby." All his life he had been subject to periodic attacks of intense cyanosis and syncope, and his activities had always been restricted. On physical examination, he was tall and well-developed with moderate cyanosis of lips and nails and pronounced clubbing of fingers and toes. The blood pressure was 103/84 mm. Hg. There was a harsh systolic murmur in the second left intercostal space transmitted widely over the precordium without thrill or diastolic component. There were no signs of congestive heart failure. X-ray of the heart (Figure 3) showed enlargement to the left. The apex appeared to be lifted above the diaphragm. An electrocardiogram showed right axis deviation. The red cell count was 8.2 million, the hemoglobin was 27.4 grams, and the hematocrit was 75.

The results of *venous catheterization* are shown in Figure 3 and Table III. It will be noted that 8 samples of blood from the right auricle, right ventricle, and pulmonary artery checked well as to oxygen content. The high systolic pressure in the right ventricle with a low pressure and narrow pulse pressure in the pulmonary artery indicated pulmonic stenosis (see Figure 4). Simultaneous pressures were not obtained from right ventricle and femoral artery, and hence the identity of these systolic pressures could not be tested. The systolic arterial blood showed a moderate degree of unsaturation. It was concluded that the patient had pulmonary stenosis and ventricular septal defect.

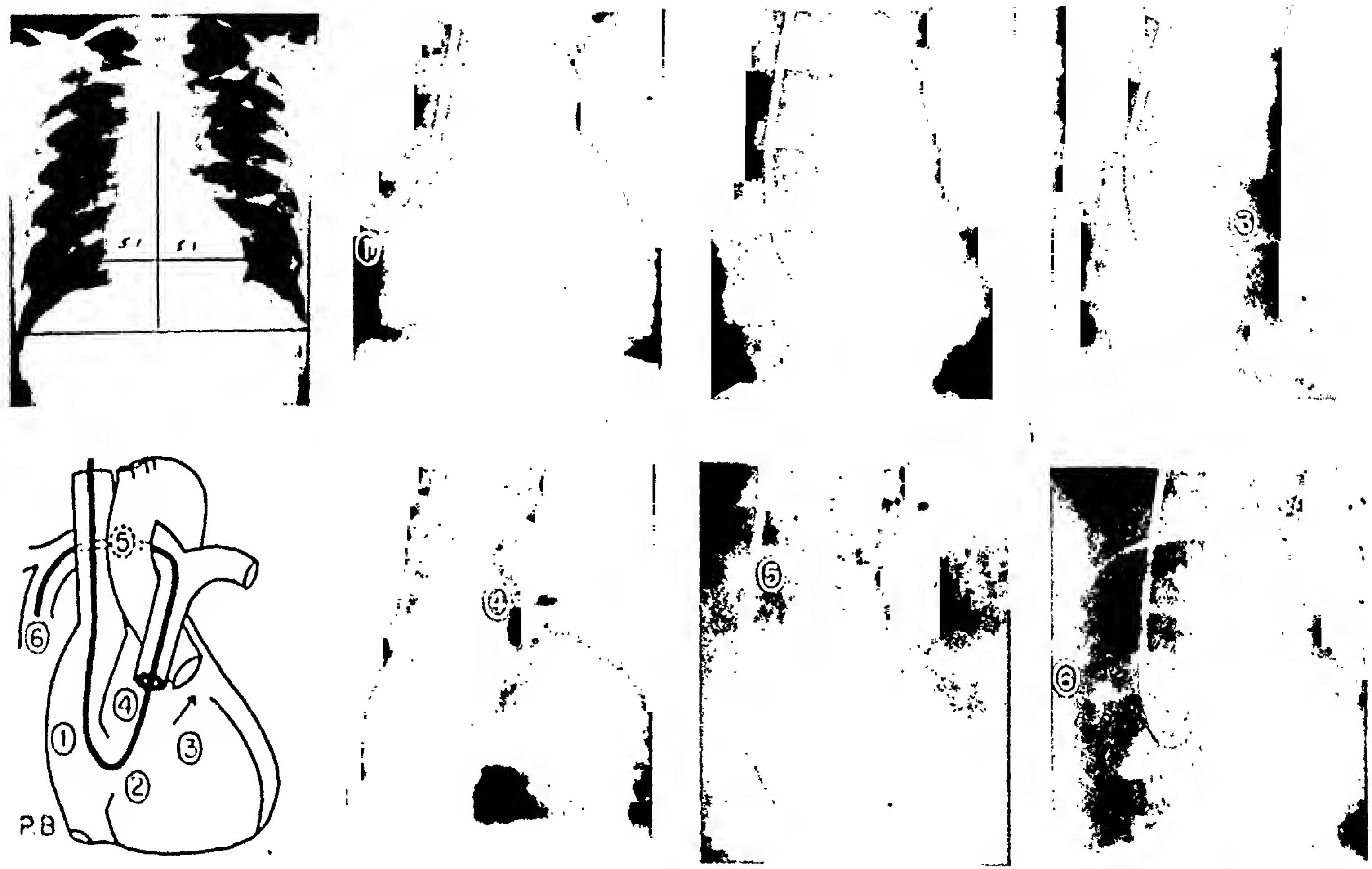


Fig. 3. Tetralogy of Fallot (Case 2)

[illegible] the catheter are identifiable by the corresponding numbers in the schema. Note that the catheter entered the pulmonary artery through [illegible] valve.

TABLE III

P. B. Age 22. Tetralogy of Fallot

Sample	Oxygen content	Pressure
	ml. per l.	*mm. Hg*
Right pulmonary artery	242	8/4
Right pulmonary artery	243	
Right ventricle (near pulmonary valve)	248	98/0
Right ventricle (mid-portion)	248	
Right ventricle (near tricuspid valve)	248	
Right auricle (lower portion)	255	0
Right auricle (mid-portion)	253	
Right auricle (upper portion)	250	
Systemic artery	295 (85 per cent)	103/80*

A–P diameter of chest	19.0 cm.
Oxygen consumption	220 ml. per min.
Body surface area	1.71 sq. m.
Pulmonary arteriovenous oxygen difference (assuming 95 per cent saturation for pulmonary venous blood)	89 ml. per l.
Peripheral arteriovenous oxygen difference	43 ml. per l.
Pulmonary artery blood flow	2.5 l. per min.
Peripheral blood flow	5.1 l. per min.
Flow through defect	
Right to left	2.6 l. per min.
Left to right	none

* Not recorded simultaneously with that of right ventricle.

Flow calculations indicated only a moderate reduction of pulmonary blood flow. Bing (4) has studied cases of tetralogy of Fallot in considerable detail by means of the venous catheter and by exercise and respiratory tests, and has evidence, both physiological and morphological, that these individuals frequently develop a collateral circulation from systemic arteries to pulmonary artery which is not revealed by application of the direct Fick principle with the venous catheter. In view of his findings, it is probably more accurate to define our flow calculations as pulmonary artery flow rather than pulmonary blood flow since we are in agreement with Bing that the method as described in this report will not reveal the collateral circulation to the lung.

Following a subsequent operation, the patient expired. *Post-mortem examination* revealed that the orifice of the pulmonary valve was only 0.5 cm. in diameter (Figure 5). A 1.5 cm. defect was present in the upper part of the interventricular septum. The right ventricular wall measured 15 mm. in thickness, and that of the left ventricle, 12 mm. There was dextroposition of the aorta.

Case 3. H. S. was a 10-year-old boy who at the age of 6 weeks was found to have a heart murmur. He had never been as active as his playmates and had had a constant cyanotic tinge to his nails and lips. He had always had dyspnea on climbing one flight of stairs, but this had not been progressive. On physical examination, he was well-developed and well-nourished. The lips and nail beds were slightly bluish. No clubbing was present. The heart was enlarged to percussion. The only murmur heard was a loud machinery murmur, maximal in the aortic region but audible over the entire precordium. There was no thrill. The blood pressure was 110/70 mm. Hg. There were no signs of cardiac failure. The hemoglobin was 17.3 grams, and the hematocrit was 49. Circulation time with magnesium sulfate was 8 seconds. An electrocardiogram showed right axis deviation. X-ray (Figure 6) and fluoroscopy of the heart showed it to be enlarged to the left with dilated engorged hilar vessels. The aortic knob projected to the right.

On *venous catheterization* the catheter passed into the right ventricle and thence into the aortic arch and the descending aorta (Figure 6). This demonstrated the existence of an over-riding aorta or a ventricular septal defect and also of a right-arched aorta. Oxygen and pressure data are

TABLE IV

H. S. Age 10. Tetralogy of Fallot and patent ductus arteriosus

Sample	Oxygen content	Pressure
	ml. per l.	*mm. Hg*
Aorta	187 (84 per cent)	110/70
Right ventricle (near pulmonary valve)	173	110/4
Right ventricle (near tricuspid valve)	140	
Right auricle (mid-portion)	130	4
Right auricle (upper portion)	120	

A–P diameter of chest	17.5 cm.
Oxygen consumption	166 ml. per min.
Body surface area	1.22 sq. m.
Pulmonary arteriovenous oxygen difference (assuming patent ductus arteriosus as only source and 95 per cent oxygen saturation of pulmonary venous blood)	25 ml. per l.
Peripheral arteriovenous oxygen difference	62 ml. per l.
Pulmonary blood flow (through patent ductus arteriosus assuming pulmonary atresia)	6.7 l. per min.
Peripheral blood flow	2.7 l. per min.

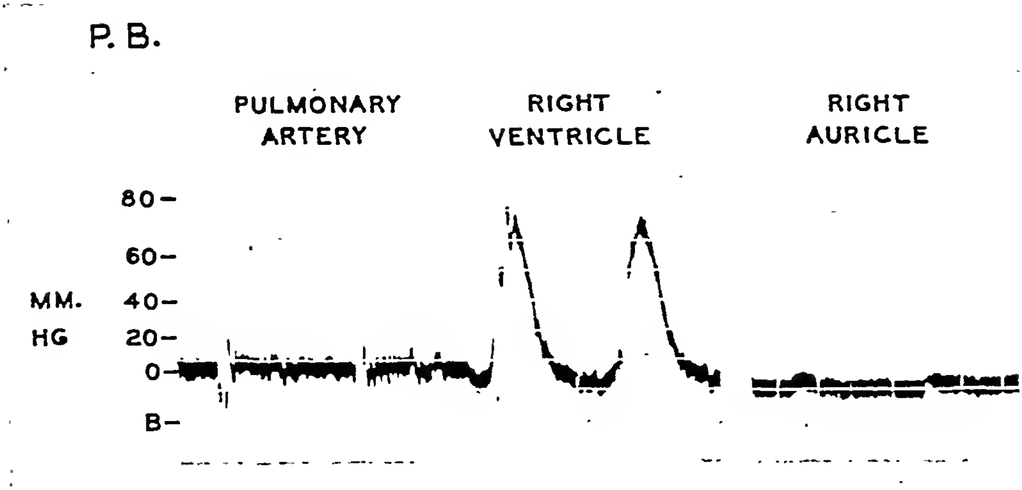


Fig. 4. Blood Pressures Obtained in Case 2

A continuous pressure was obtained as the catheter was withdrawn from the pulmonary artery to the right ventricle. Note the high systolic pressure in the right ventricle as compared with that in the pulmonary artery indicative of pulmonic stenosis.

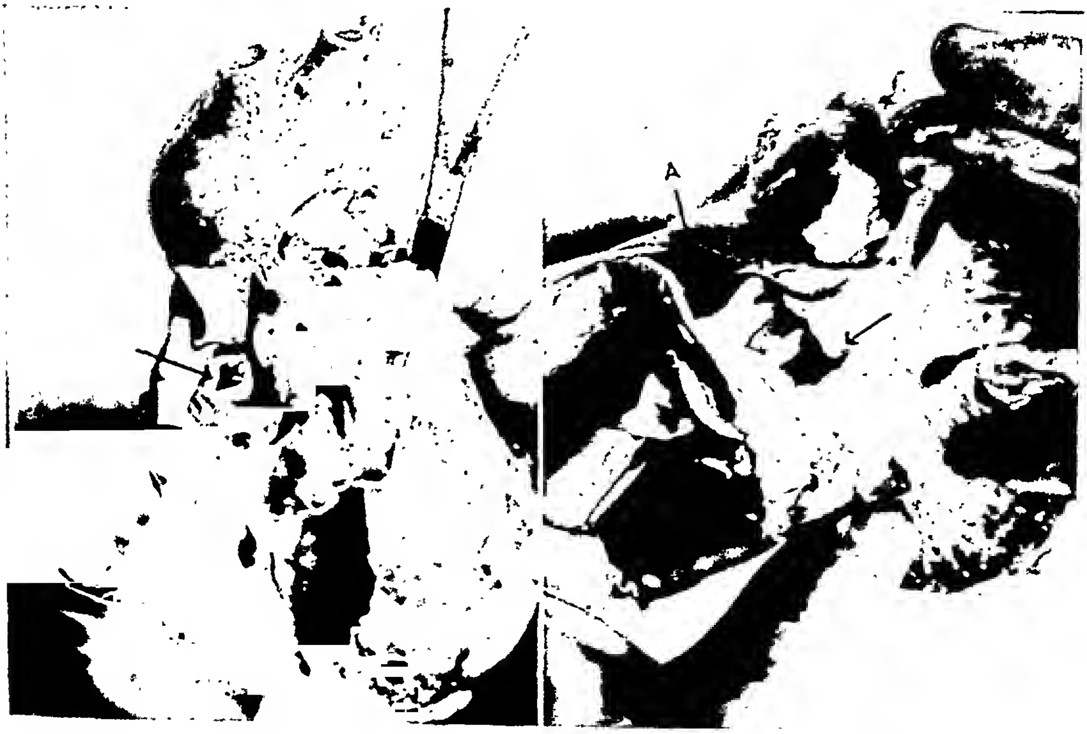
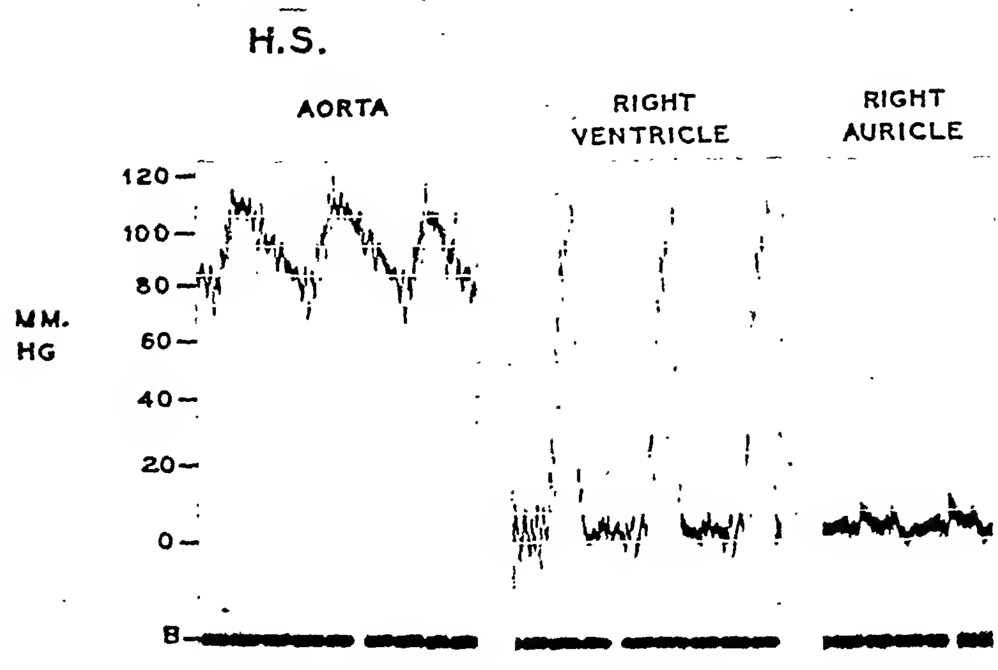


Fig. 6. Tetralogy of Fallot (Case 3)

The positions of the catheter are identifiable by the corresponding numbers in the schema. Note that the catheter passed from the right ventricle into the ascending aorta and followed the arch of the aorta to the right.


H.S.
AORTA
RIGHT VENTRICLE
RIGHT AURICLE
MM. HG
120
100
80
60
40
20
0
8

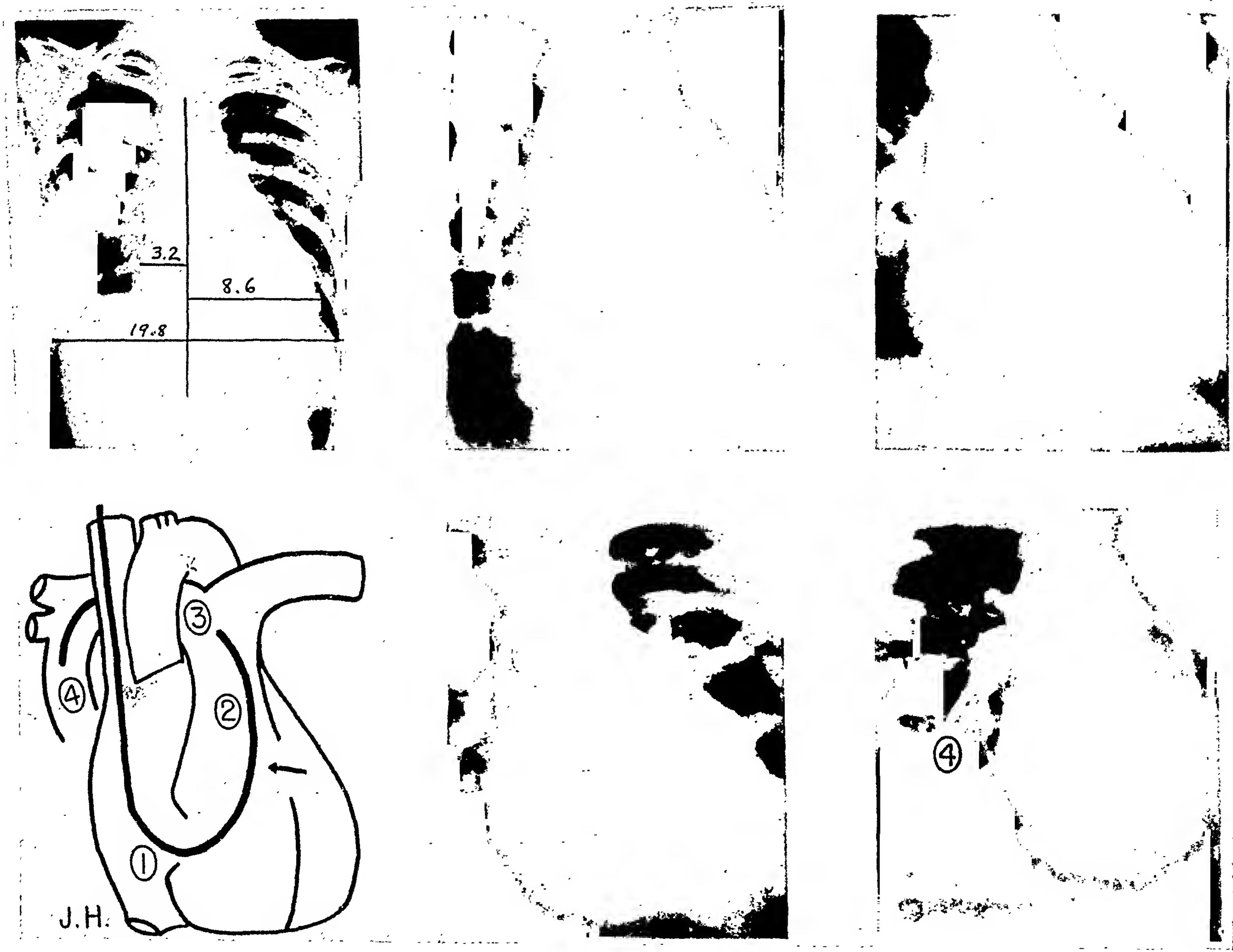


Fig. 8. Interventricular Septal Defect (Case 4)
The positions of the catheter are identifiable by the corresponding numbers in the schema.

In no case as yet has the catheter been introduced into both the aorta and the pulmonary artery in the same patient.

Interventricular septal defect (Roger's disease)

Defects in the ventricular septum vary in size and are most commonly located at the base of the heart just below the aortic valve. Since the pressure in the left ventricle is higher than that in the right ventricle, the shunt is from the left to the right side of the heart except under certain special conditions.

Case 4. J. H. was a 6-year-old boy who had had a known murmur since the age of 11 months but had never had any limitation of activity. Physical examination revealed a healthy-looking active boy of normal development. There was no cyanosis or clubbing. Cardiac findings included a blood pressure of 105/45 mm. Hg, a heart enlarged to left and right, and an extremely loud and harsh systolic murmur heard maximally at the third left intercostal space and transmitted over the entire precordium. This murmur was accompanied by a thrill. A soft early diastolic murmur was heard along the left border of the sternum. X-ray (Figure 8) and fluoroscopy of the heart showed marked enlargement, especially in the region of the left ventricle, a dilated left auricle posteriorly, and enlargement of the pulmonary artery. Hilar vessels were engorged but could not be demonstrated to pulsate. An electrocardiogram was normal.

Venous catheterization was performed as shown in Figure 8, and the results are tabulated in Table V. It is apparent that a considerable amount of arterial blood entered the right ventricle, suggesting the presence of a defect in the ventricular septum. The recorded pressures were normal. Catheterization did not explain the low diastolic pressure or the diastolic murmur. There was no evidence of a concomitant patent ductus arteriosus with pulmonary insufficiency. It was believed that this patient probably had an aortic insufficiency in addition to an interventricular septal defect.

Discussion: The recognition of interventricular septal defect by venous catheterization should depend upon finding a significant increase in the amount of oxygen in the right ventricle as compared with that from the right auricle. Two such cases have been described by Baldwin, Moore, and Noble (5). A significantly elevated oxygen content of blood in the right ventricle may reflect the presence of an interventricular septal defect or, theoretically, of a patent ductus arteriosus with an associated pulmonic insufficiency. Three instances of the latter have been suspected, in one of whom division of the ductus eradicated all murmurs and subsequent venous catheterization failed to reveal any significant variations of oxygen in the right auricle, right ventricle, or pulmonary artery. It is believed, therefore, that a diagnosis of interventricular septal defect in the presence of a patent ductus arteriosus should be made with caution.

TABLE V

J. H. Age 6. Ventricular septal defect, aortic insufficiency

Sample	Oxygen content	Pressure
	ml. per l.	*mm. Hg*
Right pulmonary artery (mid-portion)	160	29/12
Pulmonary artery (at bifurcation)	154	
Right ventricle (near pulmonary valve)	158	29/4
Right ventricle (mid-portion)	155	
Right ventricle (near tricuspid valve)	156	
Right auricle (near tricuspid valve)	126	4
Right auricle (upper portion)	121	
Superior vena cava	128	
Systemic artery	177 (100 per cent)	

A–P diameter of chest	14.5 cm.
Oxygen consumption	158 ml. per min.
Body surface area	0.82 sq. m.
Pulmonary arteriovenous oxygen difference	20 ml. per l.
Peripheral arteriovenous oxygen difference	52 ml. per l.
Pulmonary blood flow	7.9 l. per min.
Peripheral blood flow	3.4 l. per min.
Flow through shunt	4.5 l. per min.

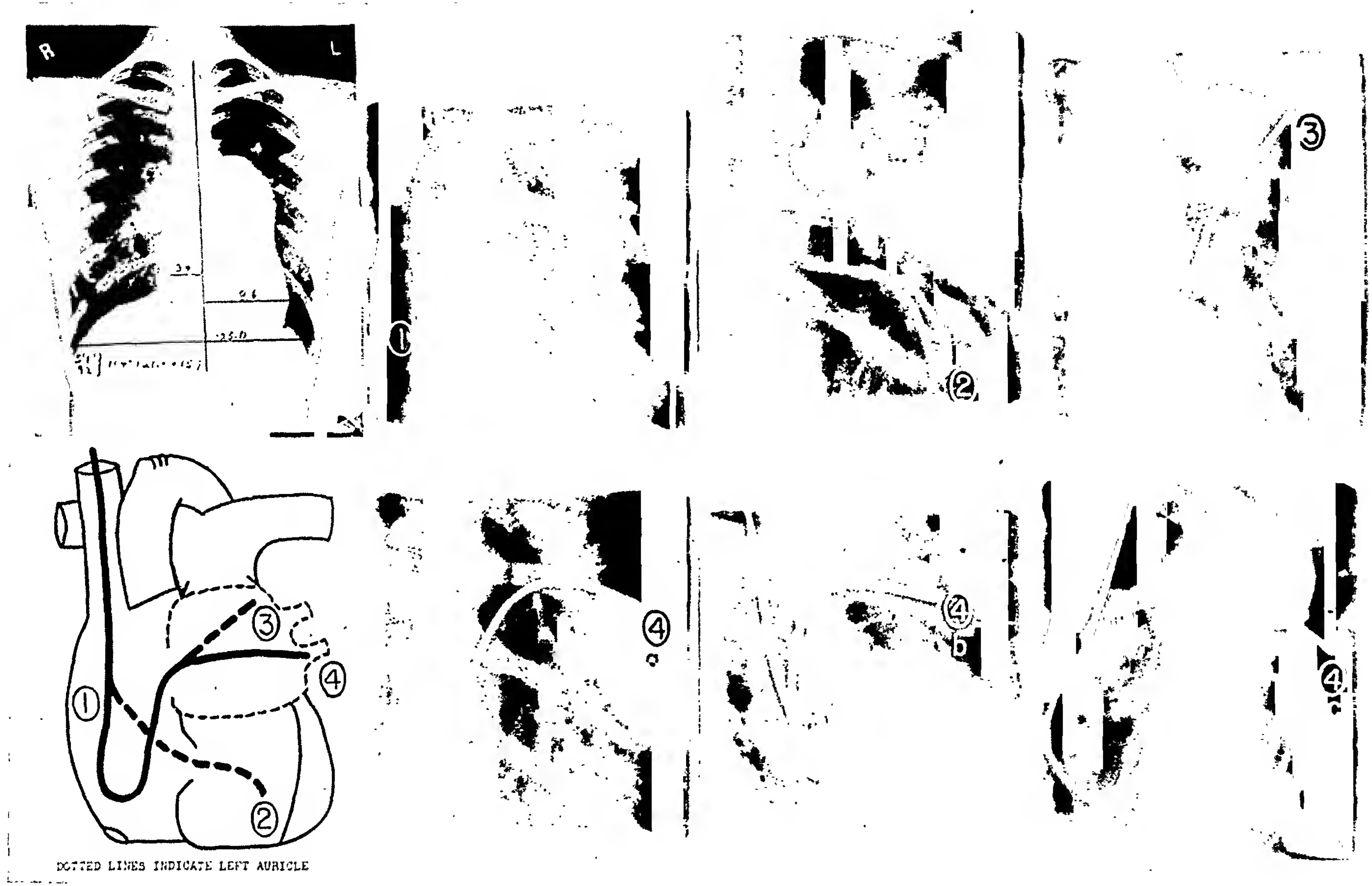


FIG. 9. AURICULAR SEPTAL DEFECT (CASE 5)

The positions of the catheter are identifiable by the corresponding numbers in the schema. Note that the catheter assumes an acutely oblique upward course in the right auricle and that the tip lies outside the cardiac shadow. The right anterior oblique view is shown in Figure 4-B, and the left anterior oblique view in Figure 4-C.

ure, the right ventricular pressure has been normal in all of our cases to date.

Interauricular septal defect

Defects in the interauricular septum vary considerably in size. The flow of blood is predominantly from the left auricle to the right auricle (6). If the defect is large or if pressure in the right auricle becomes elevated, a right-to-left shunt may likewise be present (7).

Two patients will be described to illustrate 2 different ways in which venous catheterization may aid in the recognition of auricular septal defects.

Introduction of catheter through the defect: Case 5. E. M. was a 25-year-old woman who had been found to have a heart murmur in grade school. She had always experienced fatigue rather than dyspnea on exertion. For 5 years she had had cyanosis on exposure to cold and on exertion. She was slender and there was no clubbing, but the fingers and lips were perceptibly cyanotic. The heart was overactive, and there was a harsh diastolic murmur without thrill at the fourth intercostal space. The blood pressure was 108/76 mm. Hg. X-ray (Figure 9) and fluoroscopy of the heart revealed it to be markedly enlarged with a huge pulmonary artery. An electrocardiogram showed right axis deviation.

Venous catheterization was incomplete in this patient. The catheter entered the right auricle (Figure 9), and followed a course acutely upwards and to the left so that the tip lay outside the cardiac shadow. Blood obtained from this point was 97 per cent saturated with oxygen (see Table VI). It was concluded that the catheter had passed through an auricular septal defect into the left auricle and into one of the pulmonary veins. Attempts to introduce the catheter into the pulmonary artery were unsuccessful.

Left-to-right shunt: Case 6. H. C. was a 56-year-old man who denied ever having had any symptoms referable to the heart. On admission to the hospital for carcinoma of the bladder, it was noted that his heart was enlarged to left and right, that the rate was rapid, and that there was a harsh systolic murmur without a thrill maximal at the third left intercostal space and widely transmitted over the precordium and back. No diastolic murmurs were heard. The pulmonic second sound was accentuated. Cyanosis and clubbing were absent. There were no signs of congestive failure. The [illegible] and [illegible] were normal. X-ray (Figure 10) and fluoroscopy of the heart showed it to be considerably enlarged, especially to the left, with [illegible] enlargement. An electrocardiogram [illegible].

TABLE VI

E. M. Age 25. Auricular septal defect

Sample	Oxygen content	Oxygen saturation	Pressure
	ml. per l.	*per cent*	*mm. Hg*
Superior vena cava	130	60	—
Inferior vena cava	156	72	—
Right auricle (along right wall)	142	66	6
Left auricle	196	91	10
Pulmonary vein	212	97	14
Systemic artery	187	89	108/76

Venous catheterization (Figure 10 and Table VII) revealed a large increase of oxygenated blood in the right auricle as compared with that in the superior vena cava, as well as a value in one of the samples from the right auricle practically identical with that found in the femoral artery. On the basis of these findings, the diagnosis of an auricular septal defect seemed justified. There was no evidence of other cardiac defects.

TABLE VII

H. C. Age 56. Auricular septal defect

Sample	Oxygen content	Pressure
	ml. per l.	*mm. Hg*
Right pulmonary artery	146	
Left pulmonary artery	145	35/10
Left pulmonary artery	140	
Right ventricle (near pulmonary valve)	142	35/2
Right ventricle (mid-portion)	142	
Right auricle (near tricuspid valve)	113	
Right auricle (mid-portion)	137	2
Right auricle (upper portion)	[illegible]	
Superior vena cava	107	
Superior vena cava	[illegible]	
Systemic artery	[illegible] 92 per cent	[illegible]

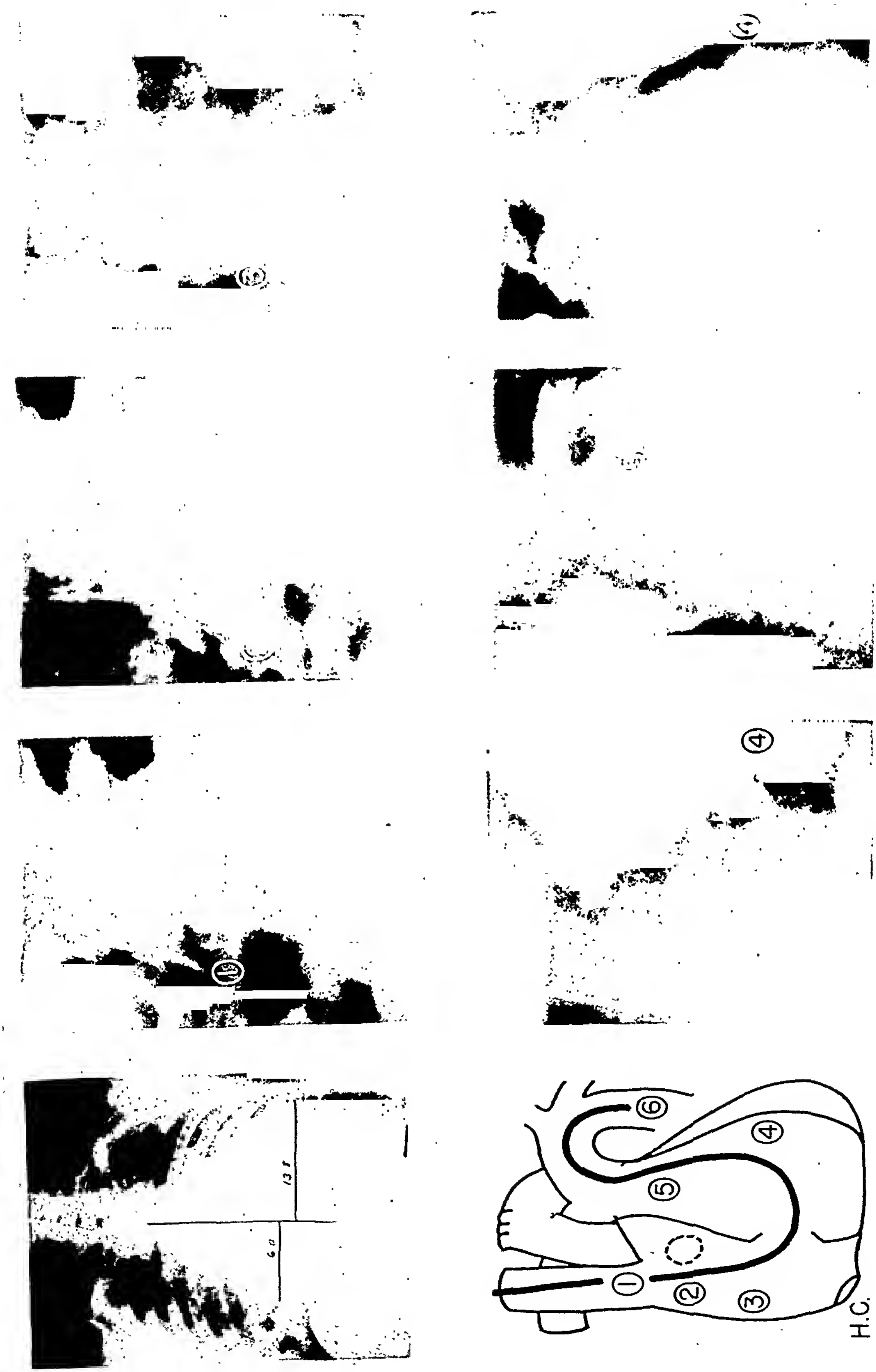


Fig. 10. Auricular Septal Defect (Case 6)
The positions of the catheter are identifiable by the corresponding numbers in the schema.

Discussion: Two methods, as described by Brannon, Weens, and Warren (9), can be utilized with the venous catheter to assist in the diagnosis of auricular septal defect: (*a*) Introducing the catheter through the defect (Patient 5), and (*b*) finding a significant increase in the oxygen content of blood in the right auricle (left-to-right shunt) (Patient 6).

Attempts to introduce the venous catheter through a defect in the auricular septum have been unsuccessful in the majority of cases. Anatomically, there appears to be no reason that, with experience, it could not be done with fair regularity.

A significant increase of oxygen content of blood in the right auricle may be present as the result of an interauricular septal defect with a left-to-right shunt, of an anomalous pulmonary vein emptying into the right auricle (7, 10), or of tricuspid insufficiency associated with interventricular septal defect. Baldwin, Moore, and Noble (5) have observed an instance of the latter and recognized tricuspid insufficiency by analysis of the auricular and ventricular pressure tracings as described by Cournand and associates (11). The recognition of a pulmonary vein emptying into the right auricle is practically impossible with our present knowledge. Statistically, auricular septal defect is much commoner than anomalous pulmonary veins emptying into the right auricle or tricuspid insufficiency associated with a ventricular septal defect. In control patients, the oxygen content of the right auricle has been observed to be as much as 1.9 volumes per cent higher than that of the superior vena cava (2). It is apparent that sizable auricular septal defects can be easily overlooked unless the catheter can be placed in the stream of arterial blood flowing from left auricle to right auricle.

DISCUSSION

Venous catheterization has opened new possibilities for the recognition of many congenital defects, for elucidating the hemodynamic changes, and for assisting in rendering a prognosis by defining the nature and physiological magnitude of the defects. As with other methods, it has its limitations and these we have tried to point out. The tetralogy of Fallot may be recognized with a high degree of accuracy if the assumptions on which the interpretations of pulmonary stenosis and ventricular septal defect were made are accepted. Patent ductus arteriosus can be detected in most instances where the defect is of sufficient size to produce symptoms or signs. Defects in the auricular and ventricular septa offer greater difficulties in diagnosis than the other 2 lesions. The diagnostic accuracy of the method in septal defects depends upon the success with which the tip of the catheter can be placed in the stream of arterial blood flowing into the right side of the heart from the left.

The necessity of obtaining multiple samples from each chamber is apparent, and a prerequisite of successful venous catheterization in patients with congenital cardiac defects is the gathering of as much information as possible by history, physical examination, x-ray, and fluoroscopy before catheterization is performed. Although we have not as yet used the method of Robb and Steinberg (12) of visualization of the cardiac chambers and great vessels with diodrast, information derived from this procedure should serve as a valuable adjunct to venous catheterization.

Finally, the increase of knowledge of hemodynamics acquired by techniques such as this will lead to increasing ease of diagnosis by simpler methods. Since certain varieties of congenital heart disease can be relieved or cured by surgery, precise diagnosis is of such importance that all doubtful cases should be studied with all available diagnostic techniques.

SUMMARY

1. Venous catheterization has been applied as a diagnostic measure and as a method of studying the hemodynamics of various types of congenital heart disease.

2. In patent ductus arteriosus, blood in the pulmonary artery is more highly oxygenated than that in the right ventricle.

3. In interventricular septal defect, blood in the right ventricle is more highly oxygenated than that in the right auricle.

4. In the tetralogy of Fallot, pulmonary stenosis is identified by finding a higher systolic pressure in the right ventricle than in the pulmonary artery. In some instances, the catheter may be introduced through the ventricular septal defect into the aorta. Other aspects of the diagnosis are discussed.

5. Auricular septal defect may be recognized by introducing the catheter through the defect into the left auricle, or by finding arterial blood in the right auricle in cases with a left-to-right shunt.

6. The limitations and potentialities of the method are discussed.

The authors are indebted to Dr. Samuel A. Levine for his help and encouragement in this study. Patients 1, 3, and 4 were referred by him for evaluation by venous catheterization, and Dr. Paul D. White sent us patient 5.

The authors wish to express their appreciation to Miss Barbara Jacobs for her technical assistance.

BIBLIOGRAPHY

1. Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Seibel, R. E., and Evans, J. M., Studies of congenital heart disease. I. Technique of venous catheterization as a diagnostic procedure. J. Clin. Invest., 1947, 26, 547.
2. Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Sagerson, R. P., and Evans, J. M., Studies of congenital heart disease. II. The pressure and oxygen content of blood in the right auricle, right ventricle, and pulmonary artery in control patients, with observations on the oxygen saturation and source of pulmonary "capillary" blood. J. Clin. Invest., 1947, 26, 554.
3. Eppinger, E. C., Burwell, C. S., and Gross, R. E., The effects of the patent ductus arteriosus on the circulation. J. Clin. Invest., 1941, 20, 127.
4. Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., Physiological studies in congenital heart disease. II. Results of preoperative studies in patients with tetralogy of Fallot. Bull. Johns Hopkins Hosp., 1947, 80, 121.
5. Baldwin, E. deF., Moore, L. V., and Noble, R. P., The demonstration of ventricular septal defect by means of right heart catheterization. Am. Heart J., 1946, 32, 152.
6. Bedford, D. E., Papp, C., and Parkinson, J., Atrial septal defect. Brit. Heart J., 1941, 3, 37.
7. Abbott, M. E., Atlas of Congenital Heart Disease. New York, Am. Heart Assoc., 1936.
8. Adams, W. E., Thornton, T. F., Jr., and Eichelberger, L., Cavernous hemangioma of the lung (arteriovenous fistula). Report of a case with successful treatment by pneumonectomy. Arch. Surg., 1944, 49, 51.
9. Brannon, E. S., Weens, H. S., and Warren, J. V., Atrial septal defect. Study of hemodynamics by the technique of right heart catheterization. Am. J. M. Sc., 1945, 210, 480.
10. Brody, H., Drainage of pulmonary veins into right side of heart. Arch. Path., 1942, 33, 221.
11. Cournand, A., Lauson, H. D., Bloomfield, R. A., Breed, E. S., and Baldwin, E. deF., Recording of right heart pressures in man. Proc. Soc. Exper. Biol. & Med., 1944, 55, 34.
12. Robb, G. P., and Steinberg, I., A practical method of visualization of the chambers of the heart, the pulmonary circulation, and the great blood vessels in man. J. Clin. Invest., 1938, 17, 507.

A BACTERIOLOGIC STUDY OF THE FACTORS AFFECTING THE EFFICACY OF STREPTOMYCIN THERAPY OF URINARY TRACT INFECTIONS [1, 2]

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Streptomycin appears to be the most useful antibiotic available at present for the treatment of infections due to gram-negative bacilli. The absorption, distribution, and excretion of the drug following various modes of administration have been studied (1). The results of clinical trials of the drug in various types of infections have been reported (2). Treatment schedules and the influence of various factors in determining the optimum dosage of streptomycin have been investigated clinically, and the results are being reported elsewhere (3). The observation that the most common cause of therapeutic failure was the development of bacterial resistance or fastness [3] to streptomycin focused attention on the necessity for further bacteriologic studies in relation to concentrations of streptomycin. The cases of urinary tract infection afforded an opportunity for further investigation of the effect of streptomycin on bacteria.

MATERIALS AND METHODS

Bacteriologic methods

In each case an attempt was made to detect all the flora involved. Urine was collected by catheterization in all females and in many of the males; in the remainder of the males voided specimens were collected after thorough cleansing of the glans and meatus with a 1:1,000 solution of mercuric chloride. After a small amount of urine had been voided to wash out the urethra, the stream was interrupted with a sterile test tube for the collection of the specimen.

The urine was centrifuged and smears were made and stained by the Gram method. Cultures were made routinely on beef extract broth, blood agar slants or plates adjusted to a pH of 7.5., and desoxycholate plates. Individual colonies were transplanted to differential sugars for further identification. When the presence of organisms which required special bacteriologic methods was suspected, the necessary conditions of partial or complete anaerobiosis or increased carbon dioxide tension were achieved.

Therapy was usually started as soon as gram-negative bacilli were detected on a smear or culture, without awaiting complete identification. During therapy, cultures were usually made daily. One or more additional cultures were done at least 24 hours after the completion of therapy, before the patient was discharged from the hospital, and a culture was made on each follow-up visit.

In vitro *sensitivity* (*susceptibility* [4]) tests

In all cases the susceptibility of the etiologic agents to streptomycin, penicillin, and sulfadiazine was determined *in vitro* when the organisms were initially isolated. A test tube method was adopted because of its simplicity and convenience. Three milliliters of beef extract broth was placed in each tube, and the tubes were sterilized in an autoclave. Sterile aqueous stock solutions were prepared which contained respectively 2.5 mgm. of sulfadiazine, 250 units of penicillin, and 1,000 micrograms (0.001 gram) of streptomycin per ml. Three series of tubes were arranged in a rack, one series for each of the drugs. One ml. of the stock solution of the chemotherapeutic agent was mixed with the broth in the first tube of the series, and 1 ml. of this mixture was transferred to the second tube; 1 ml. of the mixture from the second tube was then transferred to the third, and so forth. In order to keep the volume constant, 1 ml. was removed from the last tube of the series and discarded. In this fashion were prepared a series of 4 tubes containing graded concentrations

[1] The streptomycin used in this study was furnished by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

[2] These studies were aided by a grant from Miss Edith Pipkin and Mrs. J. B. Pipkin.

[3] Although the term resistance has been used to denote a lack of response by the organism to the drug, the possibility of confusion with resistance (immunity) of the host would suggest that another term such as insensitivity, refractoriness, fastness, or non-susceptibility should be used when referring to the bacteria. Refractoriness or fastness would imply the development of insensitivity in a previously susceptible organism. At present this is known to develop only after exposure to the drug.

[4] The term sensitivity, when applied to the response of organisms to chemotherapeutic agents, may lead to confusion with sensitivity (allergic) of the host. Perhaps susceptibility or inhibitory level is a better term.

of sulfadiazine ranging from 64 to 1 mgm. per 100 ml., another series of 4 tubes containing 64 to 1 units of penicillin per ml., and a third series of 6 tubes containing 250 to 0.3 μg. of streptomycin per ml. A control tube of broth containing no drug was added to each series.

All tubes were inoculated with approximately the same amount of the test organism; colonies were picked from solid media, or a loop of pure broth culture was transferred. The tubes were incubated for 24 hours, and then examined for bacterial growth as evidenced by turbidity. The result was usually obvious on inspection of the tubes. The lowest concentration of the drug which inhibited growth was taken as an index to the susceptibility of the organism.

The predominating organism in each case was tested; in cases of mixed infections most of the other organisms were also tested, either singly or in combination with the predominant organism. Studies were repeated at intervals during the course of therapy. The organism originally isolated was subcultured for comparison with those recovered at various intervals after the initiation of therapy. Therapy with streptomycin was not withheld pending the demonstration of sensitivity of the organism, since one object of the study was the evaluation of the *in vitro* technique as a means of predicting the efficacy of therapy. In the early stages of the study the concentrations of streptomycin in the media were occasionally varied, so that some results for concentrations of 10 and 100 μg. per ml., as well as for the standard dilutions, are reported in the tables.

Determinations of streptomycin concentrations in blood and urine

The method of Price, Nielsen, and Welch was used without modification to determine the concentration of streptomycin in serum and urine (4). Blood samples were drawn at random during the course of therapy; the blood was centrifuged and the serum used undiluted. Single voided specimens of urine, as well as 12 and 24-hour total collections, were assayed. All urine specimens were passed through a Seitz filter prior to the determination; preliminary experiments had indicated that no appreciable loss of streptomycin occurred by adsorption on the filter. Urine which was thought to contain a high concentration of streptomycin was tested in a dilution of 1:50.

The fluid to be tested was serially diluted in test tubes with a special broth adjusted to pH 7.8. In a second series of tubes the fluid of unknown streptomycin content was replaced by a solution of streptomycin containing 10 μg. per ml. To each tube in both series 1.5 ml. of a 1:100 dilution of a 12-hour growth of *Bacillus circulans* in broth was added. The tubes were incubated overnight at 37° C.; the last tube in which no growth occurred was considered the end point. The concentration of streptomycin in the test fluid was determined by comparing the end point of the first series of tubes with that of the second.

Patients

Forty-five patients received 52 courses of streptomycin for urinary tract infections due to gram-negative bacilli. Seven patients each received 2 courses of the drug. All patients were admitted to the wards of the North Carolina Baptist Hospital [5] and were treated between March and October, 1946. The youngest patient was 4 years, and the oldest, 74 years of age. In 12 instances the infection had been present 4 weeks or less; in the remaining 40, symptoms had been present for periods ranging from 1 month to 22 years. As many patients as possible were seen in the Outpatient Department at monthly intervals after discharge; the average duration of follow-up was 2½ months, and the maximum period was 6 months.

Methods and treatment

The total daily dose varied from 0.15 to 2.0 gram; the individual doses ranged from 25,000 to 333,000 μg. each and were contained in 1 to 2 ml. of sterile physiological saline [6] or water. The daily dose was divided into 6 equal doses given intramuscularly every 4 hours day and night; the usual course of therapy was 5 days, though some patients were treated for as few as 2 days and some as many as 13 days. When the patient's condition permitted, the concentration of streptomycin in the urine was raised by restricting the fluid intake in an attempt to limit the urinary output to approximately 1 liter a day.

Early in the course of the investigation no attempt was made to regulate the pH of the urine. The importance of adequate alkalinization of urine was soon demonstrated, however, by preliminary *in vitro* experiments which indicated that the effectiveness of the same concentration of streptomycin was increased 16-fold by raising the pH from 5.5 to 8.0. Thereafter, an attempt was made in all cases to increase the pH of the urine to at least 7.4. The pH of random voided samples of urine was determined with indicator paper prepared by saturating filter paper in a 0.05 per cent solution of phenol red in 70 per cent methyl alcohol; this method was found to be reasonably accurate within 0.2 of a pH unit.

RESULTS

Susceptibility of organisms

Fifty-nine strains of 8 species of organisms were isolated. A single organism was demonstrated in 44 cases; in the remaining 8 cases, the infections were mixed and were due to 2 organisms. Those organisms which were inhibited by 16 μg. or less of streptomycin per ml.—a level which can be attained in the blood—were arbitrarily considered susceptible.

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[5] We are greatly indebted to Dr. Fred. K. Garvey, of the Department of Urology, for supervising the complete urologic study of these patients.

[6] Some evidence has been presented that saline inactivates solutions of streptomycin; water is a better diluent.

TABLE I

Susceptibility of organisms

Organisms	Before therapy										After therapy (drug-fast)		
	Number cases	Streptomycin (μg. per ml.)									Number cases	Streptomycin (μg. per ml.)	
		Susceptible					Non-susceptible						
		0.3	1	4	10*	16	64	100*	250	>250		64	>250
Aerobacter aerogenes	24	11	3	3	1	5				1	9	1	8
Pseudomonas aeruginosa	22		5	6	3	5		1	1	1	11	2	9
Escherichia coli	7	2	3		1	1							
Alkaligenes fecalis	2					1	1						
Klebsiella pneumoniae	1	1											
Proteus morganii	1		1										
Streptococcus faecalis	1							1					
Streptococcus, non-hemolytic	1							1					
Total	59	14	12	9	5	12	1	3	1	2	20	3	17

* These concentrations were used in early experiments only. In some succeeding tables these data are grouped with those for the next higher concentration.

The concentrations of streptomycin required to inhibit the organisms originally isolated are shown in Table I. The susceptibility of different species of gram-negative bacilli and of various strains within the same species varied greatly, but most gram-negative bacilli were susceptible initially to concentrations of streptomycin which can be readily attained *in vivo*. Of the organisms most frequently isolated, strains of *Aerobacter aerogenes* were slightly more sensitive than those of *Pseudomonas aeruginosa;* strains of *Escherichia coli* were intermediate in susceptibility. In view of the levels of streptomycin which can be attained in blood or urine, the variations in susceptibility to concentrations of streptomycin below 16 μg. per ml. are of doubtful clinical importance.

The most striking bacteriologic finding in the study was the development of fastness to streptomycin in organisms previously sensitive to the drug. The results of sensitivity tests using strains of organisms obtained after the completion of streptomycin therapy are also shown in Table I. All the organisms were recovered from patients in whom treatment with streptomycin had failed, and the levels recorded represent the maximum degree of resistance observed. Further testing several months later of the available strains of organisms which had been found insensitive to more than 250 μg. of streptomycin per ml. revealed the following levels of susceptibility:

Concentration of streptomycin in μg. per ml.	2,500	10,000	>10,000
Number of strains	4	2	3

The relation of bacterial fastness to the dose or concentration of streptomycin, the duration of therapy, and other factors will be discussed further below.

Susceptibility to streptomycin of the organisms originally isolated is compared with their sensitivity to sulfadiazine and penicillin in Table II. No correlation was found; organisms resistant to sulfadiazine and penicillin were susceptible to streptomycin. A comparison of the susceptibility of the organisms to sulfadiazine and penicillin was made. Most of the organisms which were refractory to sulfadiazine were found also to be resistant to penicillin, but it should be stated that all of the patients had had an unsuccessful clinical trial with at least one of these drugs before streptomycin was administered.

Comparison of the dose of streptomycin with the concentration in blood and urine

In Figure 1 the total daily dose of streptomycin is compared with the maximum level attained in the blood. The blood levels varied widely, but in general the blood concentration was found to be proportional to the size of the dose. When the data are plotted in relation to the body weight, a much closer correlation is observed.

TABLE II

Susceptibility of organisms to streptomycin compared with that to penicillin and sulfadiazine

		Streptomycin (µg. per ml.)								
		0.3	1	4	10	16	64	100	250	>250
		Susceptible					Non-susceptible			
Sulfadiazine (*mgm. per cent*)	1									
	4			1		1				
	16	5	2	3	4	1		2		
	64	7	6	6		9	1			2
Penicillin (*units per ml.*)	1				2					
	4			2			.	1		
	16			1	3			1		
	64	12	8	8		10	1			2

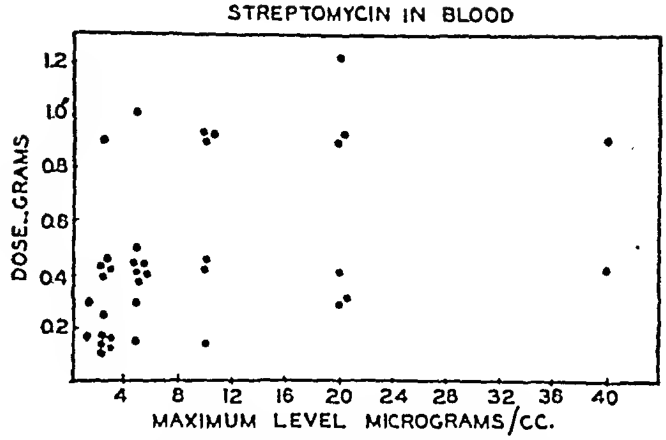

FIG. 1. COMPARISON OF THE MAXIMUM LEVEL OF STREPTOMYCIN IN THE BLOOD WITH THE TOTAL DAILY DOSE INJECTED INTRAMUSCULARLY

The maximum levels of streptomycin in the urine vary widely in relation to the total daily dose as is shown in Figure 2. The concentration in the urine was usually several times greater than that in the blood. When the maximum urinary level observed is compared with the daily dose calculated in relation to body weight, the correlation is slightly better.

Since only one of the patients exhibited marked impairment of renal function, the effect of renal

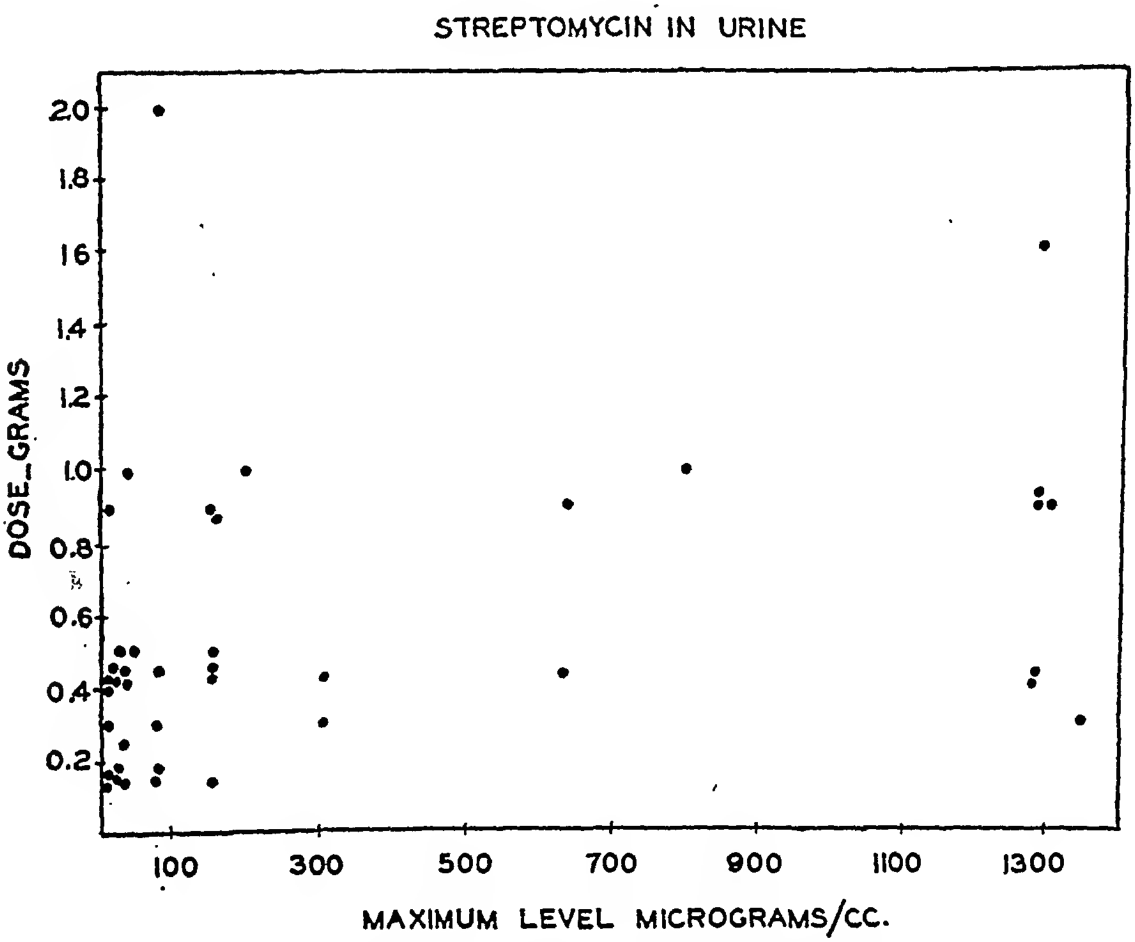

FIG. 2. COMPARISON OF THE MAXIMUM LEVEL OF STREPTOMYCIN IN THE URINE WITH THE TOTAL DAILY DOSE INJECTED INTRAMUSCULARLY

function on the concentration of streptomycin in the urine could not be determined. Alteration of the pH of the urine did not appear to increase or decrease the rate of streptomycin excretion in the few patients in whom the urinary concentration of the drug was redetermined after the administration of alkali. The greatest factor affecting the urinary concentration of the drug appeared to be the urine volume. In most cases the amount of streptomycin excreted within 24 hours in the urine fell close to the reported average of 65 per cent of the total injected daily dose (1, 5).

It appears that a minimum daily dose of 0.6 gram of streptomycin is necessary to assure a blood level of 10 µg. per ml., and that a dose of 1.5 to 2.0 grams is necessary to raise the blood level above 16 µg. per ml. With the minimum dose used (0.15 gram daily) it was possible to attain a urinary level 5 times that which inhibited susceptible organisms *in vitro*. The minimum daily dose of streptomycin required to maintain a urinary concentration above 100 µg. per ml. appeared to be 0.6 gram.

Effectiveness of therapy[7]

In 29 of the 52 infections (56 per cent), the urine became sterile before the completion of therapy.

Relation of susceptibility and streptomycin concentration to the therapeutic results. A comparison of the *initial level of susceptibility* of the organism with the maximum blood level of the drug attained indicates no correlation between these factors either in the arrested cases (Figure 3) or in the therapeutic failures (Figure 3A). In some patients infected with apparently non-susceptible organisms the urine was rendered sterile with low blood levels of streptomycin, whereas therapy failed in other cases when the maximum blood concentration exceeded many times the *in vitro* level of susceptibility of the organism.

A comparison of the initial level of sensitivity of the organism and the *maximum urinary level* of streptomycin attained reveals no correlation between these factors for either the arrested cases (Figure 4) or those in which therapy failed (Fig-

[7] Unless it is otherwise stated, the results given are based on the cultures made at discharge. The difficulty in differentiating between late relapses and reinfections is thus avoided.

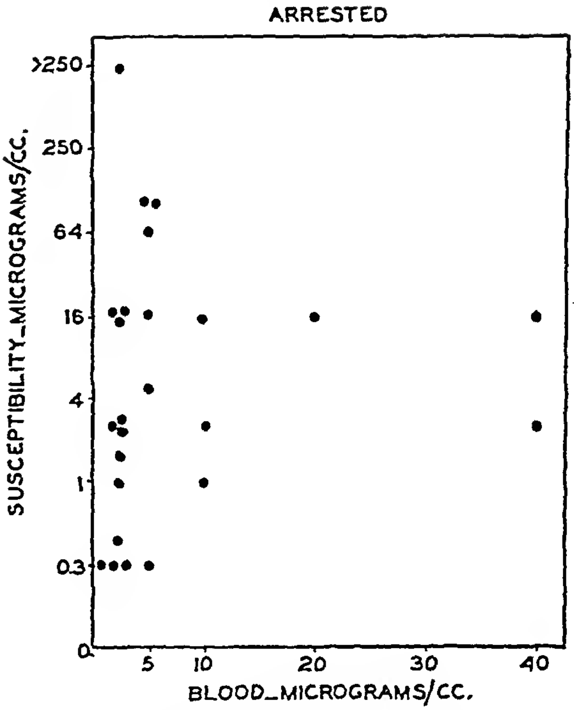

Fig. 3. Comparison of the Degree of Susceptibility to Streptomycin with the Maximum Blood Level of the Drug in the Arrested Cases

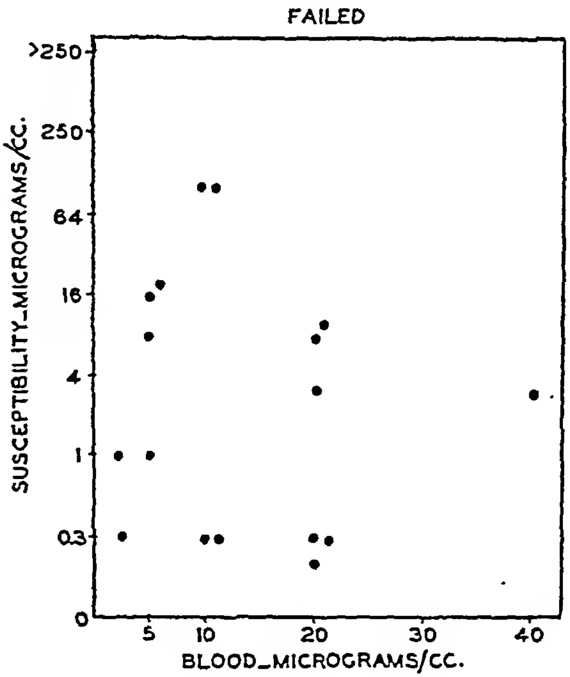

Fig. 3A. Comparison of the Degree of Susceptibility to Streptomycin with Maximum Blood Level of the Drug in the Cases Where Therapy Failed

TABLE III

Results of therapy in relation to daily dose of streptomycin

	Grams of streptomycin*								Total
	<0.20	0.4	0.6	0.8	1.0	1.4	1.6	2.0	
Number cases	11	20	3	1	12		4	1	52
Arrested	9	11	1	1	6		1		29
Failed	2	9	2		6		3	1	23

* For conciseness in this table, intermediate doses are listed as the next highest dose.

TABLE IV

Results of therapy in relation to duration of treatment

	Days												Total
	2	3	4	5	6	7	8	9	10	11	12	13	
Number cases	4	2	3	17	7	10		3	2		2	2	52
Arrested	4	1	2	10	5	6			1				29
Failed		1	1	7	2	4		3	1		2	2	23

ure 4A). Instances of favorable response to therapy were observed when the maximum urinary concentration did not exceed the *in vitro* level of susceptibility of the organism. On the other hand, failures were observed when the urinary concentration exceeded the *in vitro* level of sensitivity a hundred times. The greatest number of bacteriologic arrests occurred with a urinary concentration of 100 μg. per ml.; almost all the immediate cures occurred with a concentration of 200 μg. per ml. of urine.

Other factors affecting the therapeutic results. The *total daily dose of streptomycin* did not appear to affect the result of therapy as is shown in Table III. The results of therapy at discharge are compared with the *total duration of treatment* in Table IV. The effectiveness of streptomycin did not seem to be dependent upon the *duration of the infection* before the institution of therapy. Four chronic infections, 2 of which were mixed.

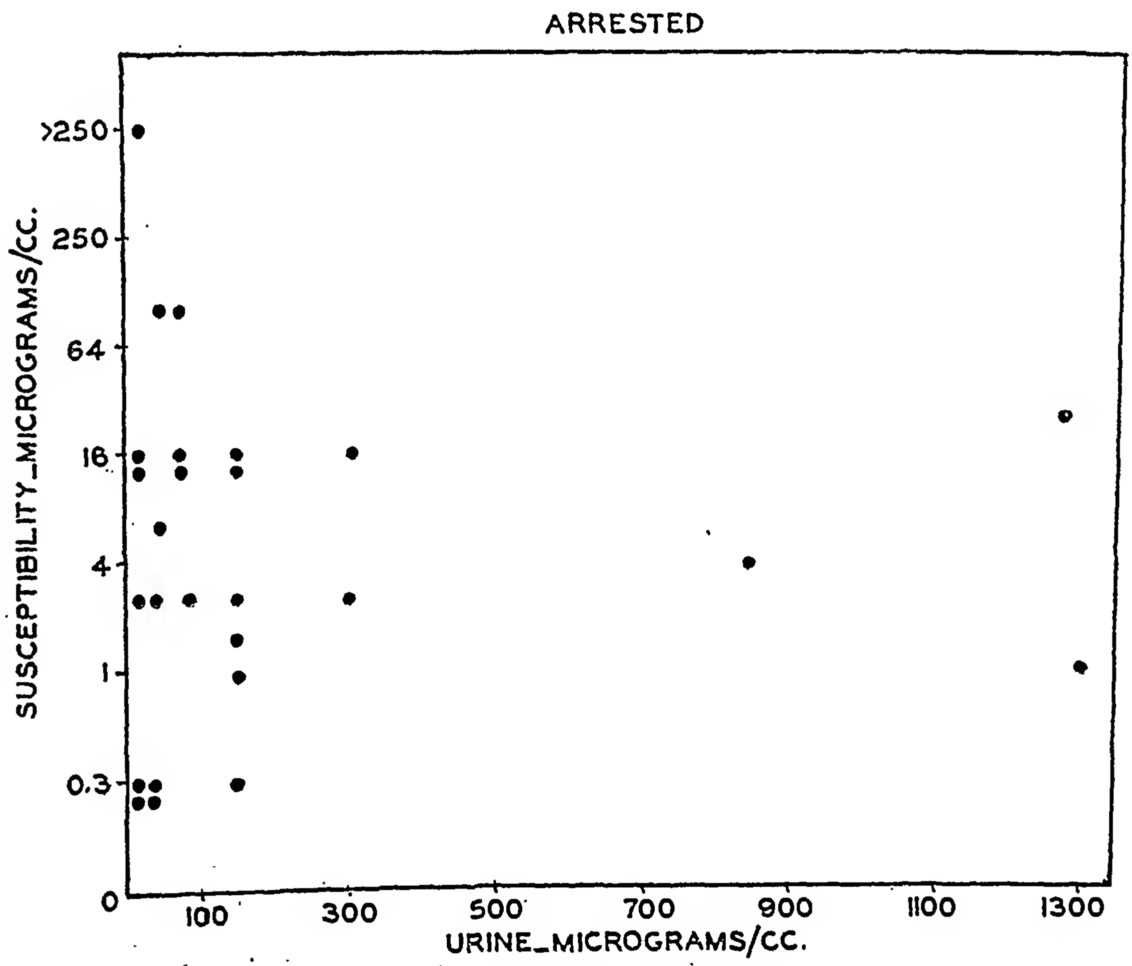

Fig. 4. Comparison of the Degree of Susceptibility to Streptomycin with the Maximum Urine Level of the Drug in Arrested Cases

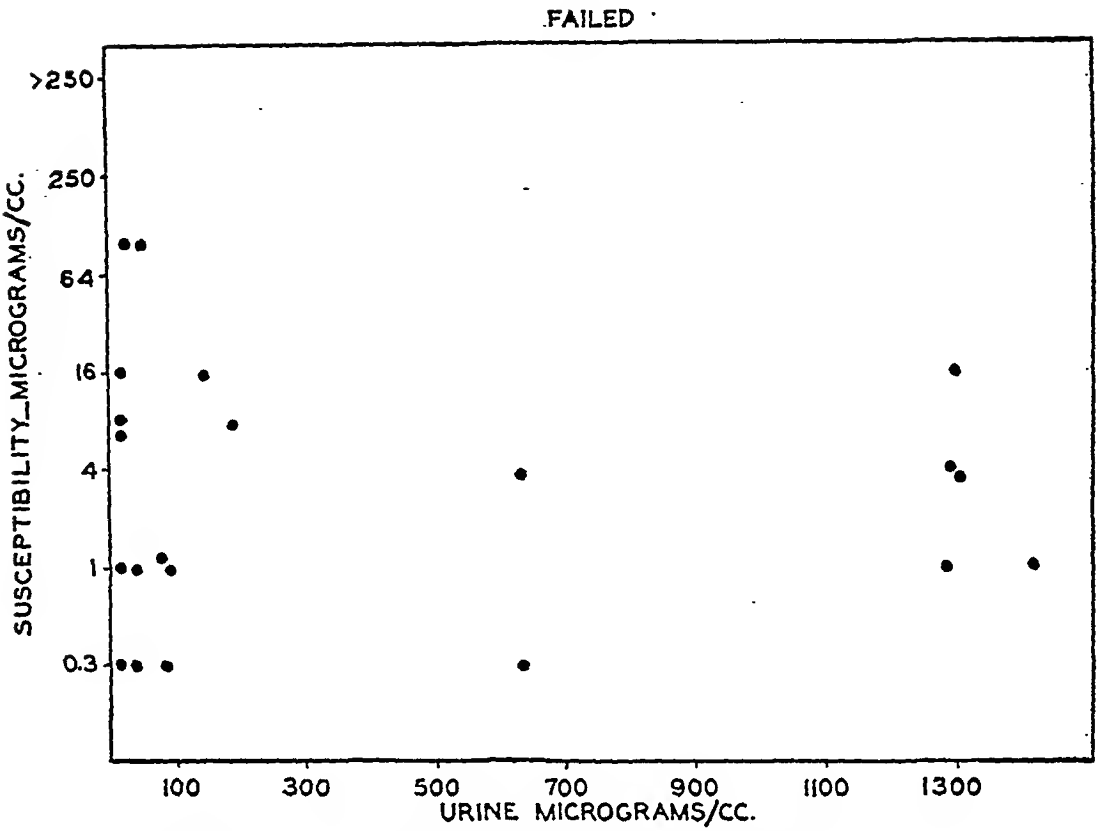

FIG. 4A. COMPARISON OF THE DEGREE OF SUSCEPTIBILITY TO STREPTOMYCIN WITH THE MAXIMUM URINE LEVEL OF THE DRUG IN CASES WHERE THERAPY FAILED

were treated for 48 hours only; the urine became sterile within 36 hours, and was sterile at discharge.

When the cases are divided into infections of the upper urinary tract (above the bladder) and those of the lower urinary tract (in and below the bladder), the *location of the infection* is seen to be of little consequence (Table V).

The effectiveness of streptomycin in sterilizing the urine did not seem to be dependent upon the *species of the etiologic agent* or its *original susceptibility* (Table VI). Of the two most commo single etiologic agents, therapy failed in 42 pe cent of the infections with *A. aerogenes* and in 5 per cent of those with *Ps. aeruginosa; A. aero genes* was slightly more susceptible in the *in vitr* test than *Ps. aeruginosa.* Because of the smal number of cases involved, the difference in re sponse is not thought to be significant. Mixed in

TABLE V

Results of therapy in upper and lower urinary tract infections with and without complications

	Uncomplicated			With complication			Total	
	Upper	Lower	Total	Upper	Lower	Total	Upper	Lower
Number cases	14	14	28	14	10	24	28	24
Arrested	14	11	25	2	2	4	16	13
Failed	0	3	3	12	8	20	12	11

TABLE VI

Results of therapy in relation to susceptibility of infecting organism

Organism		Streptomycin (μg. per ml.)						Tota
		0.3	1	4	10	16	>16	
Aerobacter aerogenes	Number cases	11	3	3	1	5	1	24
	Arrested	6	2	3	0	3	0	14
	Failed	5	1	0	1	2	1	10
[illegible]	Number cases	5	6	3	5		3	22
	Arrested	1	2	1	4		2	10
	Failed	4	4	2	1		1	12

TABLE VII

Results of therapy in cases with and without complication in relation to susceptibility of infecting organism
(Effectiveness of in vitro *test in predicting results of therapy)*

Streptomycin	Uncomplicated							With complication							Total
	Susceptible				Non-susceptible		Total	Susceptible				Non-susceptible		Total	
(*µg. per ml.*)	0.3	1	4	16	64	>64		0.3	1	4	16	64	>64		
Arrested	6	3	6	9	1	2	27	1		1				2	29
Failed				1*		1	2	5	6	4	5		1	21	23
Total				25		4	29				22		1	23	52

* Failure was due to mismanagement of therapy.

fections, including the 3 with streptococci, cleared as well as did those due to a single organism.

The effectiveness of therapy was greatly decreased by the *presence of a complication,* such as a foreign body (cystostomy tube, urethral catheter, or calculus), obstruction to the urinary flow (hydronephrosis, neurogenic bladder paralysis, or benign prostatic hypertrophy), a source of constant reinfection (prostatitis or external ureterostomy), or impaired renal function. Failures were extremely common in the presence of such complications (Table VII), and with 1 exception were accompanied by the development of drug-fastness, which is discussed further below.

Further analysis of the 23 cases in which therapy was unsuccessful revealed that in 4 cases the poor results could be at least partially attributed to *failure to alkalinize the urine;* complications were present in 2 of these cases. One patient in whom no complication was present was thought to have received an inadequate dose too intermittently, and to represent an instance of *mismanagement of therapy.* In 1 uncomplicated case the organism isolated before the institution of therapy was not susceptible *in vitro.*

Factors affecting the rapidity of bacteriologic arrest. In 28 of the 29 arrested cases the urine was sterile by the end of the third day. The rapidity with which sterilization occurred did not seem to be dependent upon the *species of bacteria.* When the data are plotted on a scatter diagram, it would appear that in general the *susceptibility* of the organism to streptomycin before therapy is inversely proportionate to the time required for

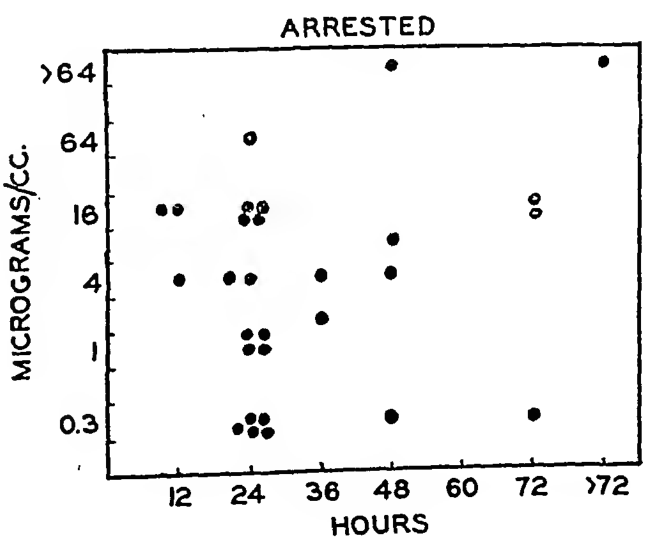

Fig. 5. Comparison of the Degree of Susceptibilty to Streptomycin with the Time Required to Sterilize the Urine in the Arrested Cases

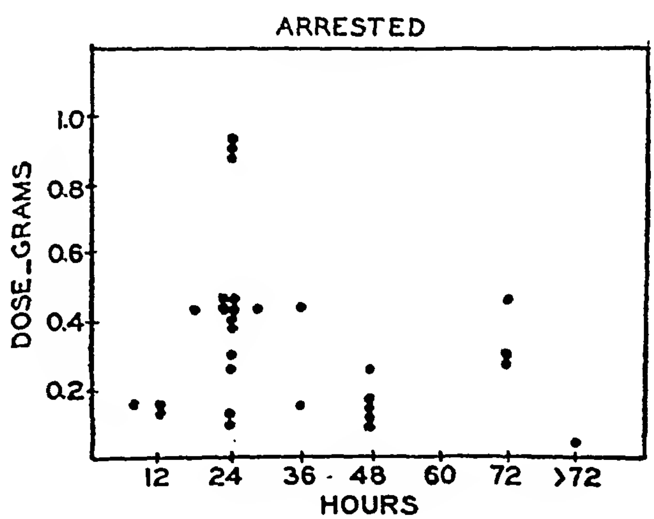

Fig. 6. Comparison of the Total Daily Dose of Streptomycin with the Time Required to Sterilize the Urine in the Arrested Cases

the urine to become sterile (Figure 5). When the *total daily dose of streptomycin* is compared with the time required to sterilize the urine, the data appear to indicate that the larger doses cleared the urine more quickly. When the data are plotted in a scatter diagram, however, the correlation between the dosage and the time at which cultures became sterile is less striking (Figure 6).

Accuracy of in vitro *test in predicting results of therapy.* The results of therapy are compared with the *in vitro* susceptibility of the organism in Table VII. The results indicate that the *in vitro* test predicted a favorable response in 24 of the 29 uncomplicated cases, and that this prediction proved to be correct in every case but one. A favorable response which would not have been anticipated occurred in 3 cases out of 5 in which the organisms were classified as non-susceptible. The 2 failures in patients without complications were attributed to mismanagement of therapy—inadequate and intermittent doses—or treatment of non-susceptible organisms. Only 2 of the patients with complications obtained a favorable response. In the remainder, with 1 exception, the organisms developed drug-fastness. The *in vitro* test would therefore appear to be useful only in cases without complications.

Follow-up studies. The majority of the patients were followed at least 1 month; the average duration of follow-up was 2½ months. Of the 29 patients whose infection was arrested at the time of discharge, 15 remained free of infection at the last follow-up examination, and 5 were found to be infected; 9 were not traced. Thus approximately ⅔ of those whose urine became sterile during therapy had sterile cultures and no pyuria when they were reexamined. In the patient with the longest history of infection (22 years), the urine was still sterile 6 months after discharge. In 3 of the 5 patients who were found to be reinfected, a different organism was cultured from the urine 3 to 8 weeks after the original infection was cleared up. Two patients whose urine became sterile under therapy were found, 5 and 8 weeks later, again to have infections with the same species of organism, which had the same susceptibility to streptomycin as the original strain; it is not known whether these cases represent reinfections or late relapses.

Fastness

The development of fastness in the organism was the most common cause of therapeutic failure observed. In 23 instances, bacilluria persisted following completion of a course of streptomycin. The prolongation of therapy did not alter the clinical results, and second courses of therapy after the organism had become fast invariably failed.

The development of fastness to streptomycin did not alter the susceptibility of the organism to other types of therapy, however. Infections with resistant organisms have cleared on subsequent therapy with mandelic acid, for instance, and have cleared without additional chemotherapy when a complicating factor—such as neurogenic bladder paralysis due to poliomyelitis—was removed.

Once an organism became fast, it remained fast. The development of refractoriness only rarely altered the cultural characteristics of the organism.

Factors affecting the development of fastness. Fastness developed much more frequently in the presence of a *complicating factor.* The fact that the organism became resistant in 3 out of 4 cases of bacteriologic failure in which the urine was not alkalinized suggested the possibility that the *pH of the urine* might have some effect on the development of fastness. Another possible explanation for failure also was present in each case, however.

The development of fastness apparently bore no relation to the *initial susceptibility of the organism* (Table VIII). Resistance developed in roughly

TABLE VIII

Initial susceptibility of organisms in relation to development of drug-fastness

	Streptomycin ($\mu g.$ *per ml.*) 0.3	1	4	16	Total
Number of organisms	14	11	10	17	52
Number developed fastness	4	5	4	5	18
Per cent developed fastness	29	46	40	30	35

30 to 45 per cent of the organisms cultured regardless of the original degree of sensitivity. In 21 of the 23 cases in which the original course of therapy was ineffective, *in vitro* tests to determine the sensitivity of the causative organism were repeated. In 1 case the organism retained its original susceptibility. In 17, it was found to be resistant to streptomycin in a concentration of at

least 250 µg. per ml. (Table I). In 3 instances the sensitivity decreased by only 1 tube in the *in vitro* test. A maximum urinary concentration of streptomycin at least 5 times as great as the original *in vitro* level of sensitivity had been obtained in all patients infected with susceptible organisms.

When the duration of therapy before the development of fastness is plotted against the initial susceptibility, it is seen that, in general, organisms which were inhibited by streptomycin in a concentration of 16 µg. per ml. became refractory earlier than organisms which were originally not inhibited by this concentration. In the majority of the cases a period of 24 to 72 hours elapsed before the organisms became fast, though, in 1 case, resistance developed in 8 hours. In several instances cultures made 10 to 24 hours after initiation of therapy were sterile, while subsequent cultures revealed resistant organisms. In only 2 instances did susceptible organisms become fast after 72 hours (Figure 7); obstruction was present in both cases, and both patients received small doses of the drug.

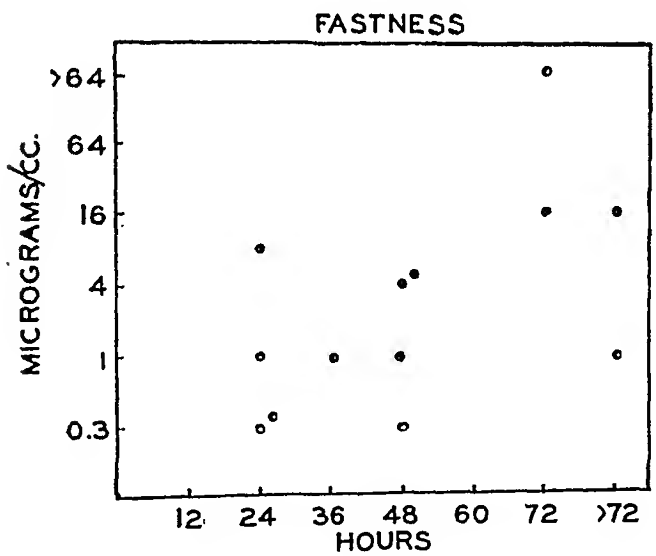

Fig. 7. Comparison of the Initial Degree of Susceptibility to Streptomycin with the Duration of Therapy before the Development of Fastness

When the duration of therapy before the development of fastness is plotted against the *total daily dose of streptomycin*, it is seen that some organisms developed resistance in 48 hours in spite of relatively large doses. On the other hand, in the patients receiving the smallest doses, fastness occasionally developed in 72 hours; one such patient was infected with a susceptible organism but also had a complication, while another was infected with an organism which was originally resistant to 16 µg. of streptomycin per ml. Small doses had been administered for 4 and 5 days to the 2 patients in whom fastness developed after the third day (Figure 8); both organisms were originally susceptible, but both patients had complications.

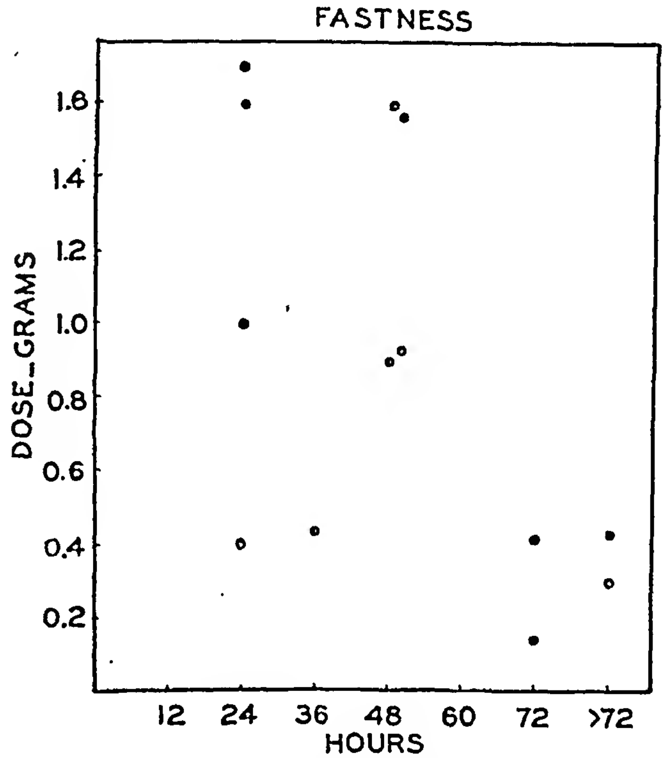

Fig. 8. Comparison of the Total Daily Dose of Streptomycin with the Duration of Therapy before the Development of Fastness

DISCUSSION

Bacteriologic methods

Most gram-negative bacilli, when isolated prior to therapy, are susceptible to streptomycin *in vitro*. The arbitrary classification of organisms into susceptible and non-susceptible strains upon the basis of the *in vitro* response to streptomycin in concentrations of 16 µg. or less per ml. is useful; this concentration can be attained in blood or urine with average doses of the drug. The fact that insensitive strains of organisms were occasionally found within the various species of organisms which are usually considered susceptible would justify preliminary *in vitro* tests in each individual case before administration of the drug.

The *in vitro* sensitivity test predicted the suc-

cess of therapy in a sufficient number of uncomplicated urinary tract infections to justify its routine use. The test was not dependable in the presence of complications, however.

Routine *in vitro* tests before therapy may conveniently be done with only 3 tubes—2 containing dilutions of streptomycin in the range of 16 μg. per ml., and another containing no drug. The organisms to be tested are introduced into one of the tubes containing streptomycin and into the tube containing no drug, which serves as a negative control; the other streptomycin-containing tube is inoculated with a susceptible organism such as *B. circulans* and serves as a positive control.

Since it is likely that increasing numbers of resistant strains of organisms will be developed, as streptomycin becomes more widely available, a dependable *in vitro* test for the detection of streptomycin-fast strains before the initiation of therapy will become an important part of the testing and identification of the organism. All organisms recovered from positive cultures after the third day of therapy should be retested for the development of fastness; if a single concentration of streptomycin is used for this purpose, it should probably be either 250 or 1,000 μg. per ml.

The finding that the sensitivity of gram-negative bacilli to streptomycin bore no relationship to their susceptibility to sulfadiazine and penicillin suggests that these drugs work through different enzyme systems of bacteria. There is a possibility that therapy with streptomycin combined with another chemotherapeutic agent might improve clinical results; further study of this question is justified.

At present there is no substance which can be added to culture media routinely to inhibit the action of streptomycin present in the body fluids of patients under therapy. The development of such a substance [8] would greatly improve the accuracy of bacteriologic methods for evaluating the effectiveness of therapy, especially in determining the time at which organisms disappear, and might aid in the earlier detection of strains developing fastness. Cultures made after therapy is begun should be incubated at least 7 days, since organisms may grow more slowly then.

[8] Hydroxylamine hydrochloride is the most promising substance in use at present.

We have no explanation for the fact that relatively few strains of Proteus were recovered from the patients in this series.

The fastness of organisms to streptomycin

Whether the development of streptomycin-fastness is due to a mutation of organisms of a single strain or to the removal of a susceptible strain inhibiting a non-susceptible strain present simultaneously cannot be stated from our data. It is known that the tolerance of cells to a toxic agent increases when they are repeatedly exposed to it. Further study from the bacteriologic point of view with the idea of potentiating the action of streptomycin in its blockage of bacterial enzyme systems, is urgently needed.

Clinically, the development of fastness in organisms is of the greatest importance. The widespread development of streptomycin-fast strains of organisms would reduce the clinical usefulness of the drug. Whether the virulence of the infecting organism is altered by the development of fastness has not been determined definitely. The early detection of resistance will prevent delay in altering therapy, thus conserving the yet small supplies of the drug, and preventing waste of money. The data suggest that the development of resistance may be avoided in some measure by the use of very vigorous therapy, from the beginning, with doses greater than the minimum ones used in this study. Infections should be hit hard when therapy is started, regardless of the duration of the infection.

Since refractoriness to streptomycin is very likely to develop in the presence of complications, and since it is a permanent characteristic of the organism once it is acquired, it might be advisable, if a complication correctable by surgical measures is present, to use other chemotherapeutic agents preoperatively and to reserve streptomycin for postoperative use. An exception should be made when the use of streptomycin might permit the performance of surgery that otherwise would not be possible.

Because of the evidence suggesting that fastness may develop in organisms highly susceptible to streptomycin if they are exposed to low concentrations for 3 to 5 days, the use of small doses of the drug to prevent reinfection in arrested cases would not seem justified and may be harmful. Reinfec-

tion is dependent on the underlying pathologic process, which is not changed by chemotherapy; late relapses or reinfections with new strains are not due to failure of the drug. Streptomycin has worked as effectively in reinfections and relapses due to susceptible organisms as in the initial infections treated; this fact leads us to suspect that the host plays no part in the development of refractoriness of the organism.

Clinical use of streptomycin

In the light of these studies it would seem justifiable at present to begin streptomycin therapy in cases of urinary tract infection as soon as gram-negative bacilli are demonstrated in smears or cultures, without awaiting complete identification of the organisms and determination of their susceptibility. As streptomycin-fast strains become more widely distributed, however, it would be wise to initiate therapy only after the determination of *in vitro* sensitivity, especially while the drug is so expensive.

From these studies it is impossible to say whether the concentration of streptomycin in the blood or that in the urine is more important. The arrest of cases of pyelonephritis would suggest that the low concentration which must be present in the glomerular filtrate and which probably is in the range of that of the blood is effective. The greater concentration of the drug in the urine below the kidney tubule is important, however; for the reduction in the number of organisms free in the urine will decrease the opportunity for reinfection of the epithelial surface of the urinary tract. It is probable that the concentration of the drug which is of the greatest importance is that in the capillaries of the kidney and the mucous membrane of the urinary tract, where immune bodies are also found.

The determination of levels of streptomycin in the blood or urine is probably not sufficiently useful to justify its use routinely. Blood and urine levels of the drug should be determined in cases which are not responding to therapy as anticipated.

The smallest daily dose used in this study (0.15 gram) was sufficient to maintain blood and urine concentrations which were found effective. Since 0.8 gram of streptomycin was required to maintain with some consistency blood and urine levels of 10 and 100 μg. per ml. respectively, this dose is suggested as the safe minimum for routine use. In view of the frequent development of fastness, however, we believe that 1.5 grams daily would be a better dose, especially in cases with complications. Therapy should be continued for 3 days. If cultures are still positive, the organism is probably fast. In a few cases cultures became negative after the third day, but this occurrence was not common. If the urine has not become sterile by the third, or certainly the fifth day, the continuation of therapy or the administration of a second course at a later time is not likely to be effective. If the culture is positive after the third day, it is probably wise to change to some other chemotherapeutic agent.

Some factor or factors other than the type of bacteria involved, the susceptibility of the organism to streptomycin, the dose of the drug administered, and the levels attained in the blood and urine are concerned in recovery; one of these factors is undoubtedly the immunity of the host. Several patients with chronic urinary tract infections were found to be hypersensitive to an intradermal test with autogenous vaccine prepared from the strain of bacteria isolated before therapy was begun.

The explanation for the decrease in pyuria and the symptomatic improvement which sometimes occurs in the presence of persistent bacilluria remains obscure. We have not been able to demonstrate decrease in motility or lysis of white blood cells in smears made on slides prepared with dried films of streptomycin in concentrations up to 1,000 μg. per ml.

SUMMARY

1. Streptomycin is the most effective antibiotic available at present for the treatment of urinary tract infections due to gram-negative bacilli.

2. The susceptibility to streptomycin of different species of gram-negative bacilli and of various strains within the same species varies greatly. *In vitro* testing for susceptibility is useful before therapy is begun and in cases where cultures remain positive after the third day.

3. Streptomycin therapy should be continued at least 3 days; if the patient has not improved after 5 days, the organism has probably become refrac-

tory and further therapy is not likely to be effective.

4. The occurrence of bacteriologic failures in this series of 52 cases could not be correlated with the susceptibility of the organism *in vitro*, the daily dose of drug, or the levels of streptomycin in blood and urine.

5. Bacteriologic failures were usually due to the presence of a complication in the urinary tract; in the presence of a complicating factor. the organism rapidly became streptomycin-fast.

6. Fastness to streptomycin develops very rapidly and to a high degree; once acquired, it is a permanent characteristic of the organism.

7. The minimum recommended dose in cases without complications is 0.8 gram daily, given in 6 equally divided intramuscular injections every 4 hours day and night; fewer failures will probably be encountered with a daily dose of 1.5 grams.

BIBLIOGRAPHY

1. Heilman, D. H., Heilman, F. R., Hinshaw, H. C., Nichols, D. R., and Herrell, W. E., Streptomycin: absorption, diffusion, excretion and toxicity. Am. J. M. Sc., 1945, 210, 576.
2. Keefer, C. S., Streptomycin in infections. J. A. M. A., 1946, 132, 4, 70.
3. Aikawa, J. K., Gillikin, C. M., Herndon, E. G., Jr., and Harrell, G. T., Simple laboratory aids for the control of streptomycin therapy in general practice. South. M. J., 1947, 40, 141.
4. Price, C. W., Nielsen, J. K., and Welch, H., The estimation of streptomycin in body fluids. Science, 1946, 103, 56.
5. Adcock, J. D., and Hettig, R. A., Absorption, distribution and excretion of streptomycin. Arch. Int. Med., 1946, 77, 179.

DIMENSIONAL, OSMOTIC, AND CHEMICAL CHANGES OF ERYTHROCYTES IN STORED BLOOD. I. BLOOD PRESERVED IN SODIUM CITRATE, NEUTRAL, AND ACID CITRATE-GLUCOSE (ACD) MIXTURES[1]

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INTRODUCTION

The value of blood, as contrasted with that of plasma or of plasma products, lies chiefly in the ability of its erythrocytes to perform their oxygen-carrying function in the circulation of the recipient, the rôle of the other formed elements being considered relatively unimportant. Studies on the storage of whole blood therefore are primarily concerned with the problem of optimal preservation of erythrocytes. Improvements of preservative technique are dependent upon reliable and practical testing procedures of the post-transfusion survival of erythrocytes. While the ability of erythrocytes to survive in the circulation of the recipient, as determined by direct measurement, remains the final criterion of the value of any method of preservation, the laboriousness of the methods available created the need for preliminary testing procedures which could be applied for screening of new preservative solutions before these are submitted to definitive testing in a direct manner. Repeated attempts have been made to use physical, osmotic, and chemical properties of stored blood *in vitro* as a guide in the appraisal of preservative methods. A short review of the existing information is indicated.

Review of the literature

The occurrence of hemolysis in stored blood was noted by the earliest observers and has become the most widely used *in vitro* test of preservation of blood. The retarding effect of glucose on the appearance of hemolysis was reported by Rous and Turner (1) and has been confirmed repeatedly. High concentrations of sucrose and dextrin (2 to 4), and decrease in blood pH (2, 4 to 6), have been shown to have similar effects. Some authors have used spontaneous hemolysis as a decisive criterion of the preservation of stored blood (7 to 9), while others have stressed the variability of this criterion and have emphasized the fallaciousness of conclusions based on its use (4, 5, 10). Particularly misleading results have been obtained with bloods preserved in electrolyte-poor carbohydrate-rich media, where the observed hemolysis after prolonged storage was incongruently slight when compared with the state of the cells determined by their survival *in vivo*. Some observers have stressed the "negative" value of the test as means of excluding rather than selecting preservative solutions (4, 11).

Changes in fragility of the erythrocytes also attracted early attention. A progressive increase of osmotic fragility was noted in bloods stored in media isotonic with respect to electrolytes (1 to 6, 9 to 15). The addition of glucose in small amounts slows the progressive increase of fragility during storage (1 to 6, 9 to 14, 16, 17). Decrease of blood pH has a qualitatively similar but less pronounced effect (2, 4 to 6). In electrolyte-poor media, fragility is often altered peculiarly, depending on the character of the preservative fluid. In De Gowin's or Rous-Turner solutions there is noted early an increased fragility which may revert to normal if the cells are washed free of excess glucose (4, 7, 10). In solutions of sucrose, dextrins, or corn syrup, substances which do not permeate the erythrocytic membrane, the cells exhibit an exceedingly low fragility, which does not change in a uniform manner during storage (1, 2, 4, 7, 10).

A gradual increase of varying degree in the corpuscular volume of erythrocytes in nearly-iso-

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Children's Hospital of Cincinnati.

tonic media has been described by most earlier observers. In electrolyte-poor media variable alterations have been found; while cells in Rous-Turner's and De Gowin's solution increase progressively to almost hemolytic (bursting) volumes, they swell only transiently and to a minor degree or may even shrink in solutions which contain dextrin, sucrose or other non-permeating carbohydrates (2, 4, 11, 17). Changes in shape and surface of erythrocytes during storage have been described (18, 19), the cells becoming more spheroidal in shape, and crenated. The increase in the mechanical fragility of erythrocytes during storage apparently bears no constant relationship to osmotic fragility, spontaneous hemolysis, or survival *in vivo* (1, 4, 7, 9).

Among the chemical properties of stored blood, changes of pH have been determined (4, 6, 20). Unfortunately most measurements have been carried out at a temperature far removed from that in the refrigerator or in the body and are therefore not easily related to actual conditions of use. It has been found that in general the pH of blood decreases during storage because of glycolysis with lactic acid formation. Liberation of ammonia (2, 21 to 23) and the exchange of potassium and sodium across the cellular membrane (2, 19, 20, 24 to 27) soon after blood is drawn have been noted by several workers. These changes appear to be somewhat retarded at lower blood pH values (19, 25). The alteration in the distribution of sodium and potassium in the cell seems reversible *in vivo* following transfusion of the stored blood (28).

Changes in the phosphorus distribution have also attracted the attention of some observers (2, 6, 11, 29, 30), who found that during storage the organic acid-soluble phosphorus of the erythrocytes decreased in concentration while the inorganic phosphorus rose. Because of the slowness of the outward diffusion great differences between cells and plasma in the concentration of inorganic phosphorus occurred. An association between the changes of the organic acid-soluble phosphorus, in particular the easily-hydrolyzable fraction, and preservability of the cells was indicated by the data (29, 30), although the observers emphasized that parallelism between the chemical change and survival *in vivo* was by no means perfect (11). Among other chemical constituents mention should be made of hemoglobin. Present evidence indicates that over long periods of storage hemoglobin maintains its oxygen-carrying capacity and that the rate of reaction between hemoglobin and oxygen is unimpaired (12, 31).

As can be seen from the summary of the information existing at present, the attempts to utilize *in vitro* changes as a measure of the behavior of the blood cells in the circulation of the recipient have led to results contradictory and disappointing to most observers. While some workers have unqualifiedly used *in vitro* tests as indices of preservation of blood on the assumption, either voiced or obviously implied, that these measurements reflected the behavior of the erythrocytes in the recipient's circulation (3, 7 to 9, 15), others, in particular those observers who used preservative solutions of high carbohydrate content, came to the conclusion that *in vitro* testing was worthless as a measure of survival of erythrocytes (4, 10, 11).

Plan of study

The study to be reported was undertaken on the basic assumption that the erythrocyte constitutes a functional unit and that therefore its physical and chemical properties are interrelated and dependent on the metabolic activity of the cell. Therefore, one would expect to find a close correspondence between suitably selected and measured physical, osmotic, and chemical indices of the cell, on the one hand, and the ability of erythrocytes to survive in the body of the recipient on the other. It was hoped that comprehensive and integrated information on the behavior of erythrocytes during storage might provide the basis for reliable *in vitro* testing procedures and for improvements of preservative technique in the light of a deeper insight into the deteriorative processes during storage. With this concept in mind a variety of measurements were carried out simultaneously and seriatim on samples of stored blood, including the dimensions of the erythrocytes, their osmotic behavior, and several chemical constituents. In the chemical studies, emphasis was put on the measurement of glycolysing ability of the cells and of correlated changes in the acid-soluble phosphorus compounds. This emphasis was based on the consideration that glycolysis is the main metabolic activity of red cells and is closely related to their acid-soluble phosphorus compounds.

In addition, studies of the changes of sodium and potassium were carried out. Recognizing that the behavior of blood cells in electrolyte-poor media may be qualitatively different from that observed in solutions near isotonicity with respect to electrolytes, it was decided to limit the first study to a comparison of the behavior of blood stored in 3 solutions, representative of the main types of nearly-isotonic preservatives in practical use at present.

Composition of preservative solutions

The solutions studied were: (*1*) simple citrate solution, (*2*) a neutral citrate-glucose solution containing glucose in a sufficient amount to raise the level in stored blood by 0.6 gram per 100 ml., and (*3*) a citrate-glucose mixture acidified by the addition of citric acid originally used by Loutit and Mollison (6, 32). The solutions were added in 15-, 20-, and 25-ml. amounts respectively, to 100 ml. of blood. For the last mixture, in this paper the term ACD–I (an abbreviation of acid citrate-dextrose), which has gained currency in the United States, will be used interchangeably with acid citrate-glucose. Table I summarizes the essential data concerning the preservative solutions used. As indicated in the table, the first 2 mixtures are approximately neutral, although it must be remembered that the pH values of such weakly buffered solutions are largely dependent on minor extraneous influences. The pH value of 5.03 for the acid preservative, which corresponds in composition to a solution of disodium citrate, may be predicted from the molar ratios of sodium citrate and citric acid present in it, on the basis of the known pK′ values of the 3 acid groups of citric acid. When this solution is added to blood, only the citric acid significantly affects the pH of blood, since at the pH range prevailing in blood, trisodium citrate has an insignificant buffering power.

TABLE I

Composition of preservative solutions

Mixture	Na citrate*	Citric acid*	Glucose*	pH	Volume added to 100 ml. of blood
	grams per 100 ml.	*grams per 100 ml.*	*grams per 100 ml.*		*ml.*
1. Citrate	3.20			(7.5)**	10
2. Citrate-glucose†	2.13		5.0	(7.5)**	15
3. Acid citrate-glucose† (ACD–I)	1.33	0.47	3.0	5.03	25

* Tri-sodium citrate $\cdot 2H_2O$, citric acid $\cdot H_2O$, glucose anhydrous.

** The pH values of these weakly buffered solutions, owing to their dependence on minor extraneous influences, are given in parentheses.

† In preparing the neutral citrate-glucose solution, sterile glucose solution was added aseptically to a sterilized bottle containing the sodium citrate solution, since heating of glucose with neutral citrate results in caramelization to a considerable degree. The acid citrate-glucose mixture (ACD–I), identical with that reported by Loutit (6), was sterilized for 30 minutes at 20 lbs. pressure in a steam autoclave, with minimal signs of discoloration.

TABLE II

Citrate, glucose and acid increments in blood after mixture with preservative solutions

Solution	Citrate		Glucose increment	Acid increment
	Blood	Plasma*		
	mM. per liter	*mM. per liter*	*grams per 100 ml.*	*meq. per liter*
Citrate	10	17	0	0
Citrate-glucose	11	19	0.65	0
Acid citrate-glucose	13	21	0.60	16.5

* The values were calculated on the assumption that citrate-ion does not permeate the cell-membrane, with an assumed volume of packed cells in blood of 45 per cent. Water shifts between cells and plasma were neglected.

In Table II are presented the calculated increments of citrate, glucose, and acid resulting in blood from mixing with the preservative solutions. In the first column is listed the citrate concentration in blood; in the next is recorded the more significant value of citrate in the supernatant plasma, calculated on the basis of the well substantiated assumption that the cell membrane is impermeable to citrate ions. The values listed are in excess of those required to suppress the effect of calcium ion on clotting, calculated by Maizels to be 10 meq. per liter (33). In the next column are given the calculated increments of glucose in blood. It may be seen that the 2 glucose-containing preservatives are like each other in this respect. In the last column is listed the amount of acid added to blood with the acid preservative solution. This amount would produce a shift in the blood pH of about 0.5 unit if no bicarbonate were displaced—a condition which undoubtedly does not prevail.

Actually, as described in a later section dealing with pH change during storage, the pH of blood was initially shifted by about 0.3 pH unit. The osmotic properties of the preservative solutions and their calculated and observed effects on blood are dealt with in a later section concerning the osmotic behavior of erythrocytes.

HANDLING OF THE BLOOD AND METHODS USED

For serial sampling, 100-ml. amounts of blood were drawn aseptically into bottles containing appropriate amounts of the preservative solution being tested. After mixing, 20-ml. portions of the blood were distributed into vials of 30-ml. capacity, similar in shape to the standard blood bank bottles. The blood samples were then placed in a refrigerator maintained at 4° C. At suitable intervals individual vials were removed for analysis. For the study of the behavior of blood under routine conditions, samples were procured from the blood bank of the Cincinnati General Hospital, where they had been drawn from donors acceptable by the standards of the blood bank, but otherwise unselected, and stored at 4° C. For testing, the bloods were transported to the laboratory under suitable precautions and sampled within 20 minutes after removal from storage.

The methods used in this laboratory for the determination of the dimensions and of the osmotic behavior of the

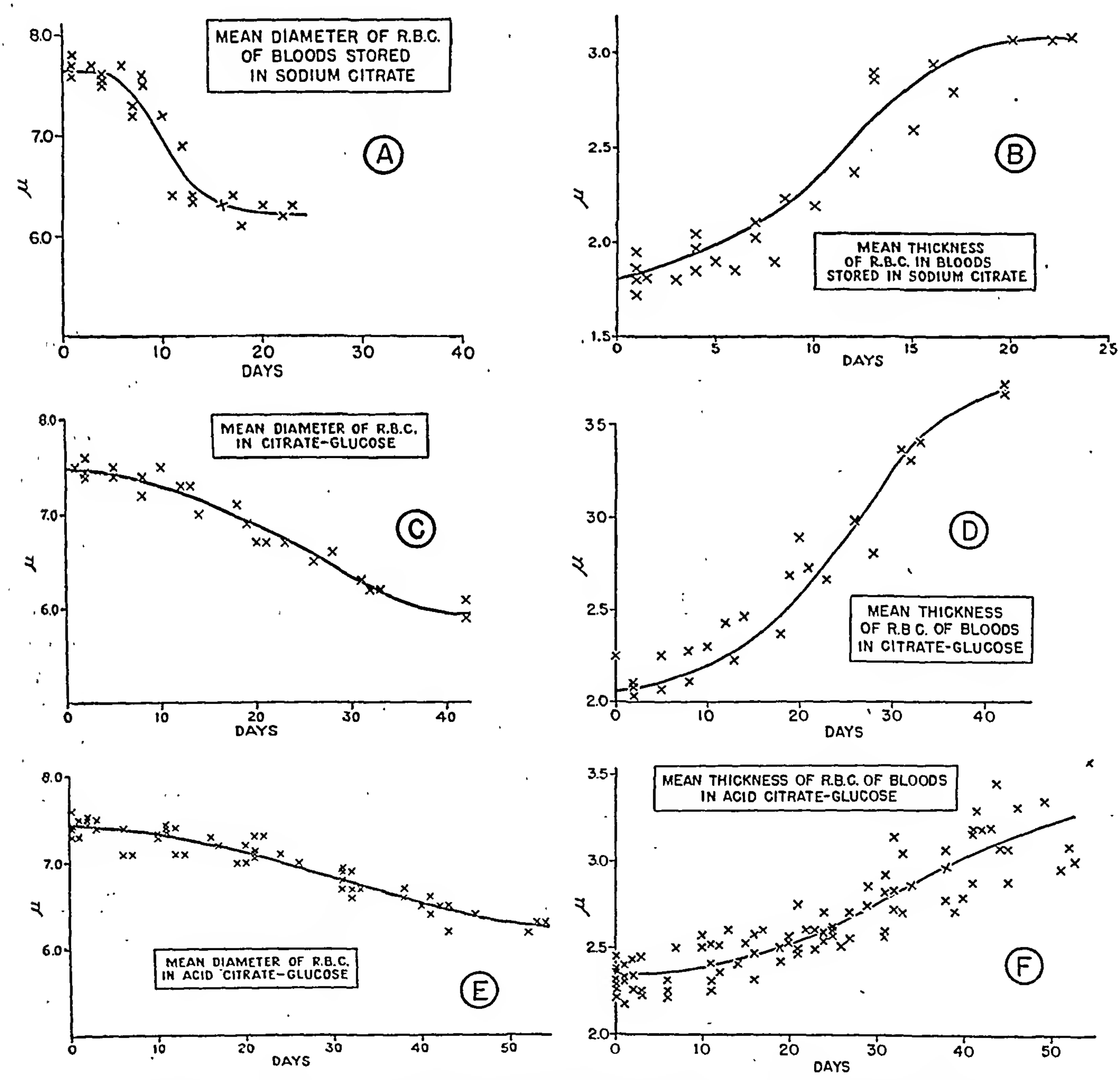

FIG. 1. DIMENSIONAL CHANGES OF ERYTHROCYTES OF BLOODS STORED IN CITRATE, CITRATE-GLUCOSE, AND ACID CITRATE-GLUCOSE SOLUTIONS

cells have been described previously (34). The chemical methods generally used excepting those for the determination of lactic acid and sugar have also been reported (35). The blood sugar was determined by the method of Nelson (36), and lactic acid by a slightly modified aeration-titration method of Friedemann *et al* (37). Reference to special methods is made in following sections, where results obtained by their use are discussed.

DIMENSIONAL CHANGES

Changes of the diameter of the cells and their thickness, calculated from the diameter and volume on the assumption of a disc-shape (34), are presented in Figure 1. (See also columns 1 and 2 of Tables III to V.) It may be seen that in all instances the diameter decreased while the thickness increased. This process is illustrated by the changes in the diameter to thickness ratio which fell from values as high as 4.1 to 2.0 during storage. The mean corpuscular volume of the cells (see column 3 of Tables III to V) rose by about 10 per cent during storage from values close to the normal in both neutral media while it did not change significantly in acid citrate-glucose solution beyond the initial rise to 115 per cent of normal. The mean surface area of the red cells (see column 4 of Tables III to V), also calculated on the assumption of a disc-shape, decreased.

TABLE III

Mean dimensions and maximal volumes of erythrocytes of blood stored in sodium citrate

Subject	Storage period	Diameter	Thickness	Volume	Surface area	Calculated volume of a sphere with same surface area	Observed maximum volume of cells	Tonicity at which maximum volume occurred
	days	μ	μ	μ^3	μ^2	μ^3	μ^3	*per cent NaCl*
J.L.	1	7.8	1.82	87	140	156	159	0.52–0.47
	3	7.7	1.80	84	137	151	158	0.52–0.47
	6	7.7	1.85	86	138	152	161	0.52–0.47
	8	7.5	1.90	84	133	144	150	0.55–0.47
	10	7.2	2.21	90	131	141	148	0.60–0.47
	13	6.4	2.86	92	122	127	140	0.60–0.47
	16	6.3	2.95	92	121	125	120	0.80–0.50
	20	6.3	3.08	96	123	128	119	0.85–0.55
	23	6.3	3.08	96	123	128	106	0.90–0.65
S.R.	0*	7.6	1.92	87	137	151		
	1	7.6	1.86	85	135	147	159	0.50–0.47
	4	7.5	1.93	85	134	146	151	0.55–0.47
	7	7.2	2.12	86	129	138	151	0.55–0.50
	12	6.9	2.37	89	126	133	129	0.65–0.52
	17	6.4	2.80	90	121	125	114	0.80–0.52
	22	6.2	3.07	93	120	123	113	0.90–0.52
E.F.	0*	7.8	1.97	94	137	151		
	1	7.7	1.97	92	141	157	170	0.52–0.45
	4	7.5	2.12	94	138	152	169	0.55–0.47
	11	6.4	3.07	97	126	133	127	0.70–0.52
	18	6.1	3.22	100	120	123	124	0.90–0.70

* Blood sample in heparin.

TABLE IV

Mean dimensions and maximal volumes of erythrocytes of blood stored in citrate-glucose mixture

Subject	Storage period	Diameter	Thickness	Volume	Surface area	Calculated volume of a sphere with same surface area	Observed maximum volume of cells	Tonicity at which maximum volume occurred
	days	μ	μ	μ^3	μ^2	μ^3	μ^3	*per cent NaCl*
S.R.	0*	7.6	1.92	87	137	151		
	2	7.4	2.03	88	133	144	149	0.55–0.47
	5	7.4	2.07	89	134	146	150	0.55–0.47
	8	7.4	2.11	91	135	147	153	0.55–0.50
	13	7.3	2.22	93	135	147	152	0.55–0.52
	18	7.1	2.36	94	132	143	141	0.60–0.52
	23	6.7	2.66	94	129	138	130	0.70–0.52
	28	6.6	2.80	96	126	133	135	0.75–0.55
E.F.	0*	7.8	1.97	94	144	162		
	2	7.6	2.10	95	141	157	162	0.55–0.47
	5	7.4	2.26	97	139	154	144	0.55–0.50
	12	7.3	2.41	101	139	154	144	0.65–0.52
	19	6.9	2.68	100	133	144	134	0.70–0.55
	26	6.5	2.98	99	127	134	129	0.85–0.60
	32	6.2	3.31	100	125	131	124	0.90–0.60
D.B.	0*	7.6	2.03	92	139	154		
	2	7.4	2.09	90	135	147	149	0.55–0.50
	8	7.2	2.28	93	133	144	148	0.55–0.47
	14	7.0	2.46	95	131	141	144	0.60–0.52
	21	6.7	2.72	96	128	136	133	0.70–0.55
	33	6.2	3.40	103	127	134	131	0.75–0.60
	42	5.9	3.70	101	123	128	127	0.90–0.65

* Blood sample in heparin.

TABLE V

Mean dimensions and maximal volumes of erythrocytes of blood stored in acid citrate-glucose mixture I

Subject	Storage period	Diameter	Thickness	Volume	Surface area	Calculated volume of a sphere with same surface area	Observed maximum volume of cells	Tonicity at which maximum volume occurred
	days	μ	μ	μ^3	μ^2	μ^3	μ^3	*per cent NaCl*
S.R.	0*	7.6	1.92	87	137	151		
	0	7.4	2.26	97	140	156	154	0.52–0.50
	3	7.4	2.24	97	138	152	157	0.52–0.50
	6	7.4	2.21	95	137	151	158	0.52–0.50
	11	7.4	2.26	97	139	154	151	0.55–0.52
	16	7.3	2.32	97	137	151	147	0.60–0.52
	21	7.1	2.47	98	134	146	145	0.60–0.52
	26	7.0	2.51	97	132	143	146	0.60–0.52
	31	6.9	2.59	97	131	141	137	0.65–0.55
	38	6.6	2.78	95	126	133	139	0.70–0.52
E.F.	0*	7.8	1.97	94	144	162		
	0	7.6	2.36	107	147	167	164	0.55–0.50
	3	7.5	2.44	105	145	164	163	0.55–0.52
	10	7.3	2.57	108	143	161	162	0.55
	17	7.2	2.60	106	140	156	141	0.65–0.52
	24	7.1	2.70	107	139	154	144	0.70–0.52
	31	6.9	2.82	105	136	149	138	0.75–0.52
	38	6.7	3.06	108	135	147	134	0.75–0.55
	46	6.4	3.29	106	130	139	125	0.90–0.65
D.B.	0*	7.6	2.03	92	139	154		
	1	7.3	2.39	100	139	154	150	0.55–0.52
	7	7.1	2.49	98	135	147	149	0.55–0.52
	13	7.1	2.56	101	136	149	145	0.55–0.52
	20	7.0	2.60	100	134	146	144	0.60–0.52
	32	6.7	2.83	100	131	141	135	0.70–0.55
	41	6.7	2.87	101	131	141	137	0.75–0.52

* Blood sample in heparin.

A comparison of the 3 preservatives with regard to the dimensions of the erythrocytes indicates that at the outset the cells of citrated blood were slightly shrunken and flatter, those of blood in acid citrate-glucose somewhat swollen and rounder, and those in citrate-glucose of about the same shape and size as were the cells of heparinized samples. During storage the values for diameter and thickness increasingly approached each other with resulting increased "roundness" of the cells. The pattern of change, with its characteristic sigmoid-shaped time curve, was essentially the same in all preservatives as was the final form attained by the cells. The behavior of the red cells in the 3 preservatives differed primarily in the length of time required for the dimensional alterations to run their course. For instance, while the cells in citrate required 10 days on the average to reach a diameter of less than 6.6 and a thickness of more than 2.8 micra, cells in citrate-glucose assumed a comparable shape in about 25 days and those in acid citrate-glucose, in about 35 days. The differences in the rates of dimensional change are illustrated more clearly in Figure 2, in which

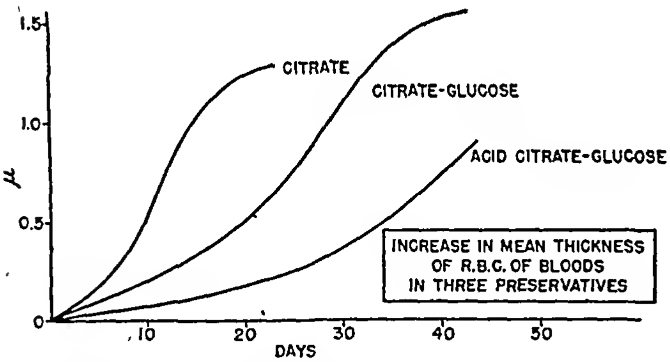

FIG. 2. AVERAGE INCREASE IN MEAN THICKNESS OF ERYTHROCYTES STORED IN 3 PRESERVATIVE SOLUTIONS

the 3 preservatives are compared with respect to the average increase in thickness of the erythrocytes above the initial value. A similar order is oberved in the changes in the calculated surface area.

The calculated decrease in the mean surface area of the cells during storage may be explained as follows: if the assumption is accepted that the mean surface area of a given cell population is constant, as suggested by several observers, the calculated decrease of the area might be apparent only; the hypothesis may be then advanced that an interrelation exists between the calculated change in surface area and the microscopically visible crenation; going one step further, one may assume that the extent of the calculated alteration in the surface area is a reflection, perhaps even a quantitative measure, of the degree of crenation of the erythrocytes.

The alterations in the dimensions of the red cell during storage may be interpreted as manifestations of increasing deterioration of the cell form, resulting in increasing roundness of the cell, or more exactly, in closer approximation of all cell diameters. In this process the cell is changed in form from a biconcave disc to the shape of a wrinkled bag. The "S"-shaped pattern of change with time in the dimensions of the cell, characteristic of phenomena of biological growth and decay, would indicate a relationship between the dimensional and functional state of the cell.

The alterations in the corpuscular volume of the cells, initially and during storage, will be discussed in detail in the following section. They appear to be determined primarily by changes in the osmotic relationships and by shifts of the pH in cells and plasma. Here, it may be pointed out that the disproportion between the increasing approximation of all cell diameters and the change of the cellular volume, seems at least partly to account for the crenation of stored erythrocytes.

OSMOTIC CHANGES

In Figures 3, 4 and 5 are presented data on the percentage of erythrocytes hemolyzed in varying concentrations of sodium chloride. The data for hemolysis in 0.9 per cent sodium chloride solution, while not a direct measure of true "spontaneous" hemolysis, *i.e.*, of the amount of hemoglobin released in the supernatant plasma, are comparable to the amounts measured in the plasma after mixing and centrifugation of stored blood. The hemolysis curves are presented as plots of percentage of cells hemolyzed in different concentrations of sodium chloride at any given time and as time curves for any given concentration of sodium chloride. Also included in the presentation are combined data on several bloods in 0.6 per cent sodium chloride solution, chosen as representative of the osmotic behavior of the erythrocytes.

In comparing the 3 preservatives with respect

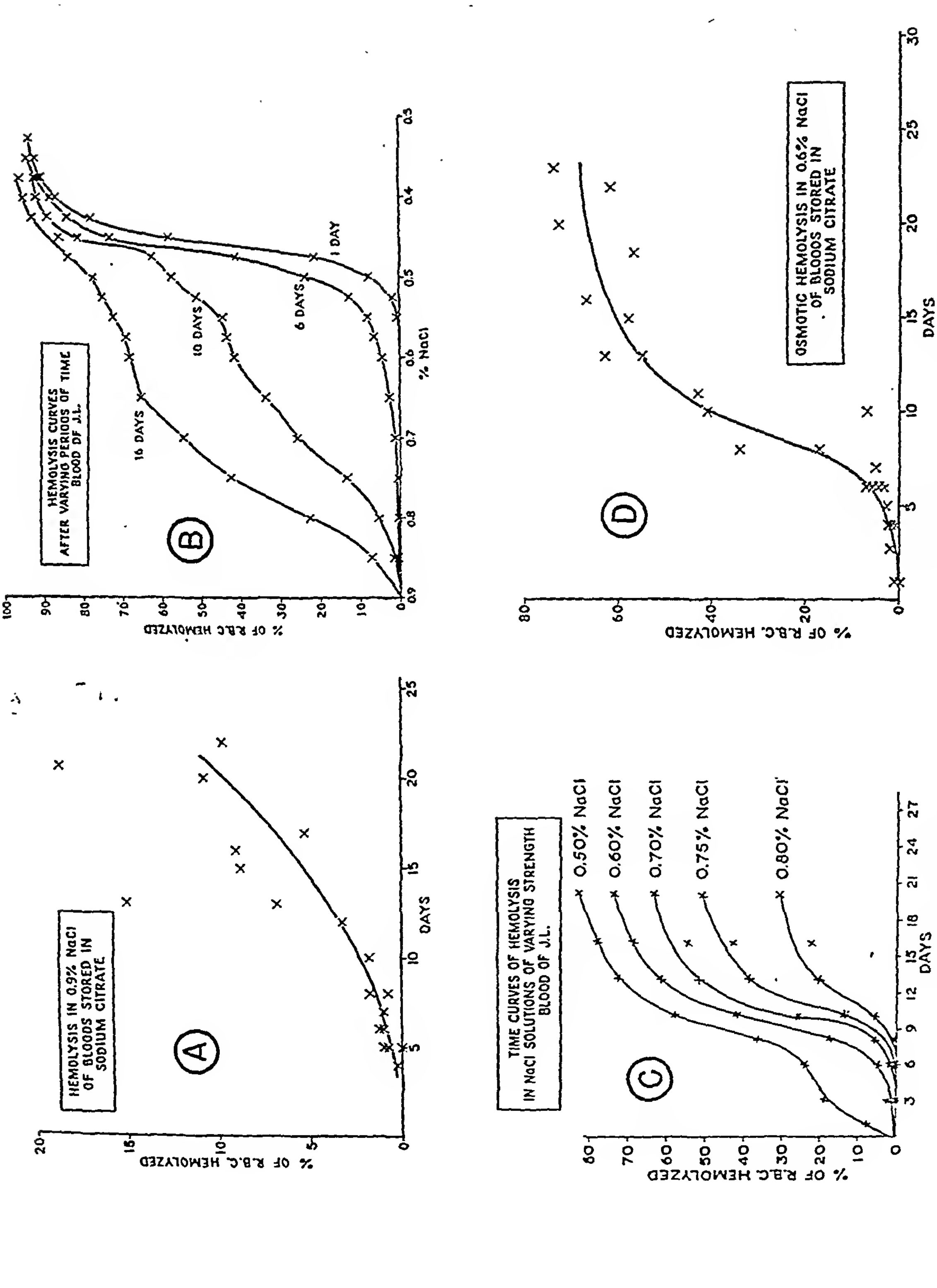

Fig. 3. Osmotic Hemolysis of Erythrocytes of Bloods Stored in Sodium Citrate Solution

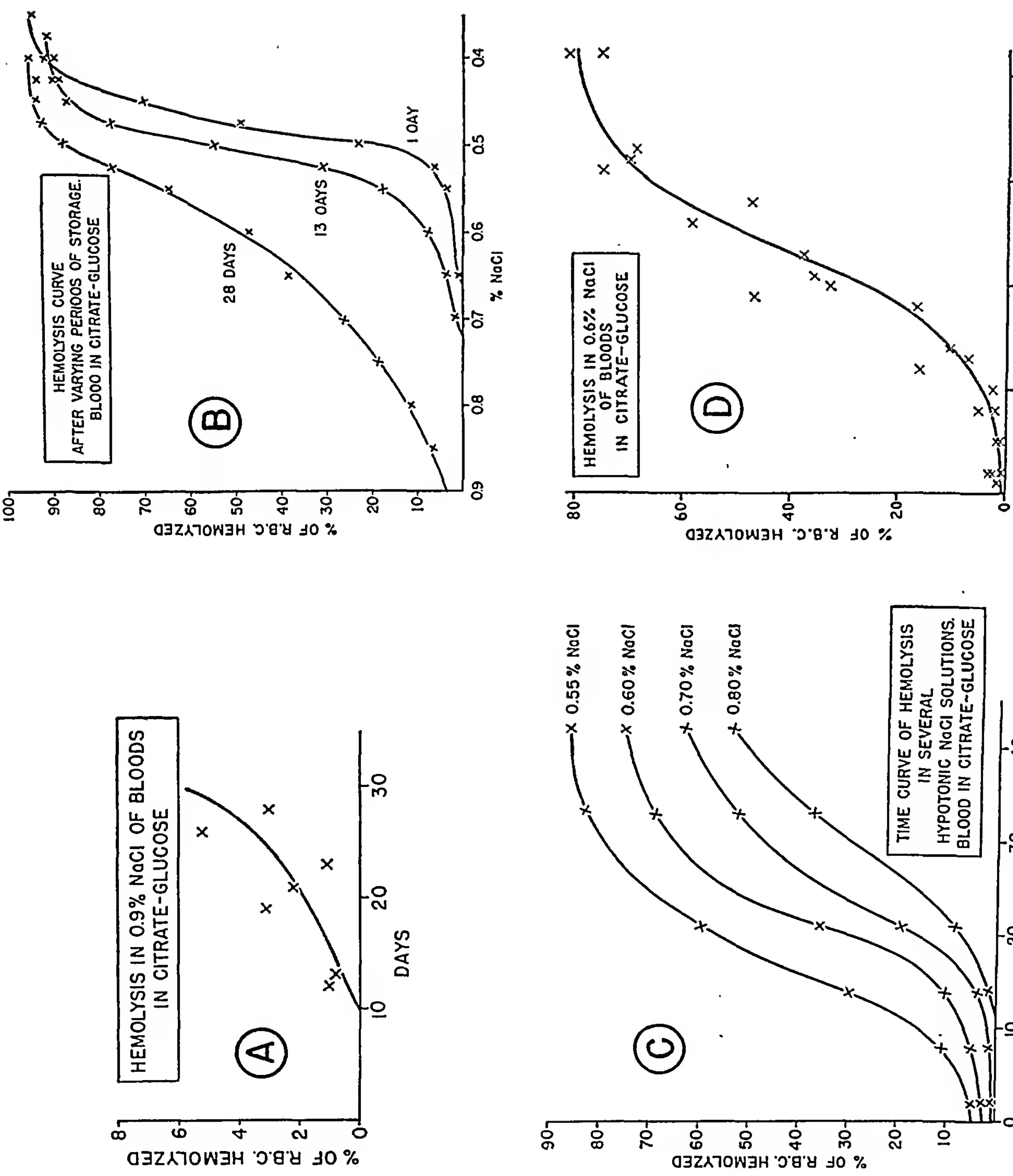

FIG. 4. OSMOTIC HEMOLYSIS OF ERYTHROCYTES OF BLOODS STORED IN NEUTRAL CITRATE-GLUCOSE SOLUTIONS

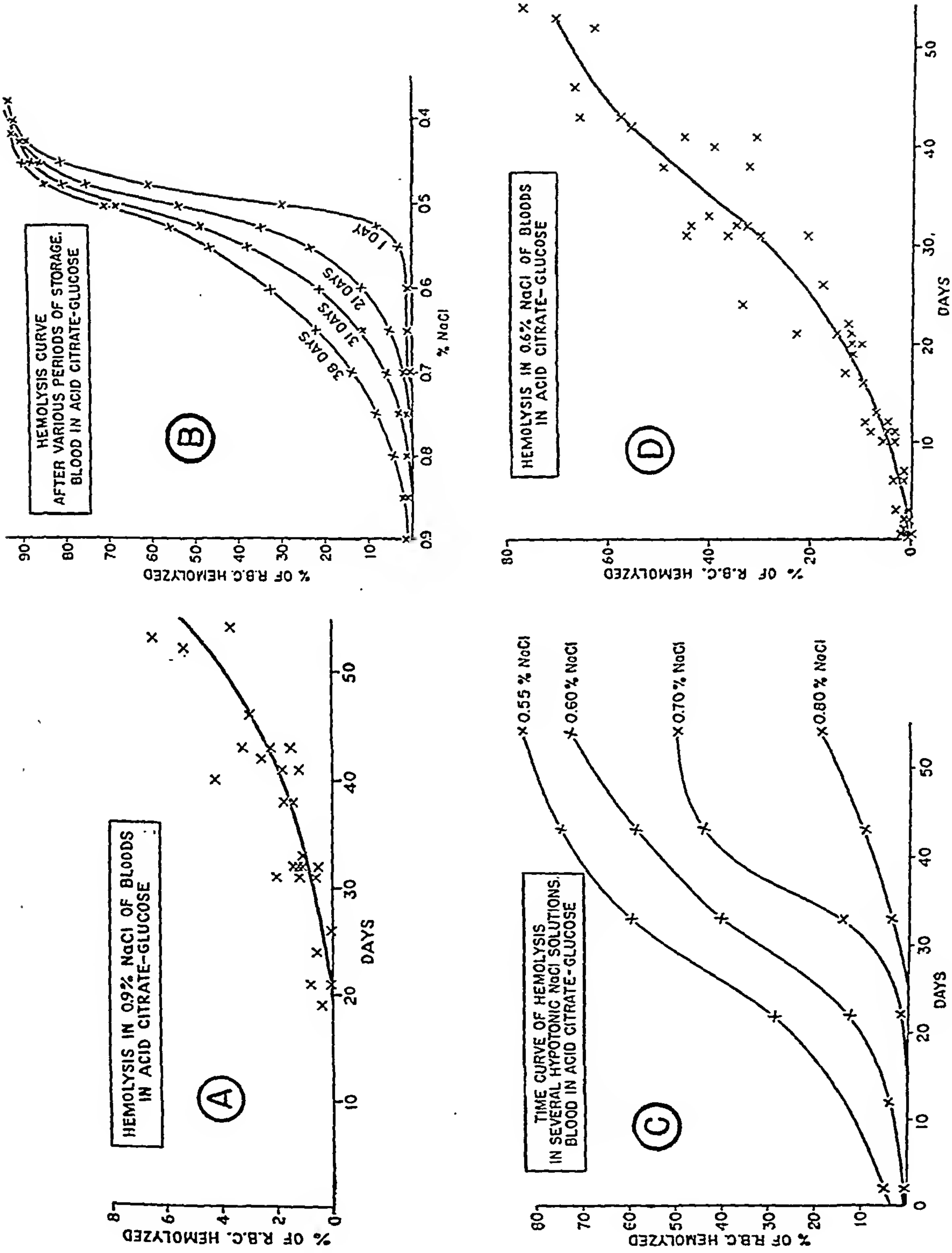

Fig. 5. Osmotic Hemolysis of Erythrocytes of Bloods Stored in Acid Citrate-Glucose Solution

to hemolysis in 0.9 per cent NaCl solution, it is evident that significant degrees of hemolysis were observed earliest and that hemolysis increased at the most rapid rate in simple citrate solution, while it was latest and slowest to increase in acid citrate-glucose.

From an inspection of the hemolysis curves it is apparent that, from being closely similar for all 3 preservatives at the outset, they changed during prolonged storage at different rates. The earliest and most rapid change occurred in the citrate and the slowest, in the acid citrate-glucose solution. An examination of the time curves of hemolysis in different hypotonic salt solutions, which lend themselves better for comparison, indicates that the 3 preservatives arrange themselves in the same order with respect to osmotic hemolysis as they did with regard to dimensional changes and hemolysis in 0.9 per cent NaCl solution.

It should be pointed out that the usual qualitative way of measuring fragility—noting only the points of beginning and of completed hemolysis—would have resulted in a highly misleading picture of the changes occurring, owing to the fact that a small proportion of the cells showed greatly increased fragility after a few days of storage, thus shifting the point of beginning hemolysis to a considerable extent, while the bulk of the erythrocytes was affected at a much later date. This consideration is of particular importance in the study of bloods collected in glucose-containing preservative solutions, where a minute fraction, less than 1.0 per cent, of the cells exhibited greatly increased fragility immediately following the collection of the blood, causing a shift in the point of beginning hemolysis to NaCl concentrations of 0.7 per cent or higher.

The data on hemolysis on 0.9 per cent NaCl solution presented here are comparable to those reported by others on "spontaneous" hemolysis (5, 7). With respect to hemolysis in hypotonic salt solutions, a pattern of change similar to that described here was observed by those workers who followed the percentage hemolysis of erythrocytes in a given salt solution during storage (5, 17, 30). However, different results were reported by those authors who used *median corpuscular fragility* (38), that is, the tonicity of a salt solution in which 50 per cent of erythrocytes are lysed, as a measure of the osmotic behavior of erythrocytes. This index is unsatisfactory because of the fact that great differences in the percentage of erythrocytes hemolyzed result from small variations in salt concentration. The *median corpuscular fragility* is susceptible to the influence of changes in temperature and pH and is affected by a number of minor variations of experimental technique (39). Workers who used this test observed marked alteration initially in bloods stored in various glucose-containing preservatives but noted also that this index changes more gradually with time in bloods preserved in acid than in neutral mixtures (4).

From a consideration of the data here presented one may exclude the hypothesis that hemolysis in 0.9 per cent NaCl solution, or so-called "spontaneous" hemolysis, can be due to osmotic factors. During storage the cells swell only by 10 per cent, far below their critical volume; and there is no relation apparent between the extent of the increase in volume and the degree of hemolysis. Other circumstances, such as lytic factors in the plasma, may account for the hemolysis of stored erythrocytes in nearly-isotonic media. The fact that spontaneous hemolysis increased with the period of storage may be explained on the assumption that the presumed lytic factors affected deteriorated cells only. If such were the case, the degree of spontaneous hemolysis would represent a measure of the degenerative processes taking place in the cells, affecting perhaps primarily the cell membrane.

Critical volume of hemolysis: Additional information concerning the process of osmotic hemolysis can be gathered from a comparison of expected and observed maximal (critical) volumes of the cells. The calculation of this cell characteristic is based on 2 assumptions: (*1*) that the cell membrane cannot be stretched, so that the mean surface area is constant for a given cell population, and (*2*) that the cells are capable of reaching the shape of a perfect sphere. Granted these assumptions, a cell could reach a maximal volume equal to that of a sphere possessing the same mean surface area. This point, the critical volume, would coincide with the beginning of hemolysis. Available data generally are in good agreement with this theory.

In column 5 of Tables III to V are tabulated for citrated blood the maximal volumes after various periods of storage, calculated on the basis of the

corresponding values for the surface area. It can be seen that during storage both observed and calculated maximal volumes, while agreeing reasonably well with each other, decreased from the initial values.

The tonicity of the salt solution at which maximum swelling occurred is tabulated in column 7 of Tables III to V. With increasing time of storage, maximal swelling took place at increasingly high concentrations of NaCl solution. The zone of maximal swelling which may be defined within narrow limits in fresh blood, became less clearly delimited and broader. On examining the data on the osmometric behavior as well as on the maximal volumes of the cells after extended periods of storage it should be remembered that they are not representative of the state of the cell population as a whole since they are descriptive only of cells remaining unhemolyzed, which by then may constitute half or less of the total cell population. In a comparison with respect to change in maximal volume of the erythrocytes, the 3 preservatives range themselves in the same order and at closely similar intervals, as they did in the preceding comparisons.

Two interpretations of the changes in the maximal volume during storage and of the coincidence of the calculated and observed figures may be offered: (*1*), that the surface area actually decreased during storage, with corresponding actual change in the maximal volume attainable by the cells and (*2*), that the apparent decrease of the surface area parallels in extent only the degree of crenation of the erythrocytes; in that case, the expected maximal volume should have remained the same throughout the storage period. The coincidence of the observed maximal volumes with the expected ones calculated on the basis of the apparent decrease of the surface area might be explained as a reflection of the loss of elasticity of the cell membrane, perhaps of its inability to unfold completely to an uncrenated form.

A comparison of hemolysis and volume change of the erythrocytes in hypotonic salt solutions with their maximal critical volumes suggests that during storage the erythrocytes became hemolyzed at volumes increasingly far below their calculated critical volumes. This behavior may signify that the cells, possibly owing to progressive deterioration of the elastic properties of the cell membrane, hemolyze before attaining the spherical shape.

In general, the osmotic properties of the erythrocytes suggest the conclusion that the functional state of the cell, perhaps more specifically that of the cell membrane, during storage becomes the limiting factor determining its behavior rather than the osmotic relationships between the cell and its surrounding medium.

Corpuscular volume: In the following, changes in the corpuscular volume of erythrocytes during storage will be considered from the standpoint of the osmotic relationships obtaining in blood. The discussion will deal with this problem under 2 headings: (*1*) the initial changes, calculated and observed, produced by the addition of preservatives and (*2*) the changes occurring during storage. In considering the first point, it may be useful to list the osmotic properties of the preservative solutions employed and their calculated effect on the electrolyte and osmotic concentration of blood. As may be seen from Table VI, the solutions varied considerably among themselves with respect to their composition: simple citrate preservative is a hypertonic solution with respect to total solutes as well as electrolytes, while citrate-glucose, although osmotically more concentrated, is actually

TABLE VI

Osmolarity of preservative solutions and resulting changes in the osmotic and electrolyte concentration of blood

Solution	Citrate	Citrate-glucose	Acid citrate-glucose
Sodium citrate, *mM. per liter*	109	73	45
Sodium citrate, *m. osm. per liter*	436	292	181
Citric acid, *m. osm. per liter*			22
Glucose, *m. osm. per liter*		278	167
Total osmolarity of solution, *m. osm. per liter*	436	570	370
Total osmolarity of solution, *per cent of initial blood tonicity*	141	184	119
Effective tonicity,* *m. osm. per liter*	436	292	203
Effective tonicity, *per cent of blood tonicity*	141	94	66
Osmolarity of blood after mixing,† *m. osm. per liter*	324	351	324
Osmolarity of blood after mixing, *per cent of initial*	104	113	104
Electrolyte concentration of blood after mixing, *per cent of initial*	104	99	92

* As effective tonicity is designated, this is the osmotic concentration of substances which permeate the cell-membrane very slowly, if at all. In this instance its value was calculated on the assumption that the electrolytes, but not glucose, conform to this condition.

† Calculated on the basis of an initial concentration of 310 milliosmols per liter of water in blood, and of a water content of 0.80 gram per gram of blood.

almost isotonic with blood in terms of effective tonicity, owing to the permeability of the cellular membrane to glucose. Acid citrate-glucose solution, which also contains a higher concentration of solutes than blood, is hypotonic with respect to its electrolyte content. The changes in the osmotic and electrolyte concentration of blood, listed in the same table, were calculated on the basis of an assumed initial water content of 0.80 gram per gram of blood, and an osmolarity of 310 milliosmols per liter of water in blood. In the first 2 columns of Table VII are listed observations on the corpuscular volume and on the cellular concentration of hemoglobin, expressed as percentage of simultaneously determined values on heparinized blood samples. It may be seen that the initial values for the corpuscular volume and the hemoglobin concentration of erythrocytes in both neutral preservatives agreed reasonably well with the predicted values, while the cells in the acid preservative were considerably more swollen. This difference is largely accounted for in terms of the Donnan theory by the additional effect of lowering of pH on the corpuscular volume of the cells. By the use of equations descriptive of the distribution of water between cells and plasma (40), acidification *per se* may be calculated to produce an increase in the corpuscular volume of erythrocytes of about 6 per cent.[2] While many of the values adopted for the calculation are approximations and while several factors have been neglected, it may be justifiably assumed that the variable of major importance is the ionic equivalency of hemoglobin which is determined by the value of cell pH. The cumulative effects of the changes in osmolarity and pH of blood in acid citrate-glucose solution would result in an increase of the corpuscular volume of erythrocytes of about 14 per cent, a value in good agreement with the observed one.

TABLE VII

Change in corpuscular volume during storage

The results represent the mean value, together with the standard error of the mean.

Solution	Storage period	Cells in own plasma: Corpuscular volume	Cells in own plasma: Corpuscular hemoglobin concentration	Volume of packed cells in 0.9 per cent NaCl
	days	*per cent of "normal"**	*per cent of "normal"**	*per cent of volume in plasma*
Citrate	0	97.6±0.6	104.0±0.4	111.7±1.4
	20–30	104.3±0.9	95.9±0.9	122.1±1.5
	Per cent change from initial value	+6.9±0.9	−7.8±0.9	+9.4±1.5
Citrate-glucose	0	99.3±1.2	101.2±0.5	111.0±1.2
	20–30	106.0±1.0	93.6±0.6	117.4±1.0
	Per cent change from initial value	+6.7±1.0	−7.5±0.6	+5.8±1.0
Acid citrate-glucose	0	113.0±0.3	89.3±0.7	100.0±0.4
	20–40	113.0±0.3	89.3±0.7	108.5±0.4
	Per cent change from initial value	0.0±0.3	0.0±0.3	+8.5±0.4

* The values in heparinized blood samples have been designated as "normal."

[2] The theoretical changes in the water content of the plasma may be calculated by means of equations 23 and 24 of Van Slyke, Wu and McLean (40).

$$(23)\quad (H_2O)_s = (H_2O)_b \times \frac{2(B)_s - (BP)_s}{2(B)_b - (BP)_s - (BP)_c + (Hb)}$$

$$(24)\quad (H_2O)_c = (H_2O)_b \times \frac{2(B)_c - (BP)_c + (Hb)}{2(B)_b - (BP)_s - (BP)_c + (Hb)}$$

in which the parentheses denote units of substance per unit of whole blood, and the subscripts, s, c, b refer to plasma, cells, and whole blood, respectively. B, P, and Hb refer to the sum of cations, the non-diffusible anions, and the osmolarity of hemoglobin, respectively. The following assumptions and substitutions were used in applying this equation: It was assumed that all factors except for $(BP)_c$, by which the equivalency of the non-diffusible anions in the red cells is designated, did not change on acidification. A numerical value of 0.84 gram per ml. was assigned to $(H_2O)_b$, the water content of whole blood. An initial cell volume of 36 per cent was assumed. To $(B)_b$, the total cations of whole blood, a value of 138 meq. per liter was assigned, and to $(B)_s$ the cations of serum, a value of 93 meq. per liter. To $(BP)_s$, the ionic equivalency of the serum proteins, a value of 10 was given, on the basis of an assumed protein concentration of 7 grams per 100 ml. of plasma. For the hemoglobin portion of $(BP)_c$, an initial value of 16 was calculated on the basis of an assumed hemoglobin concentration of 5 mM. per liter, a cell pH of 7.20, and an iso-ionic point of reduced hemoglobin of 6.81 at 38° C. For the buffering power the value of Adair (41), 9.2 meq. per mol. of hemoglobin was adopted. The ionic equivalency of hemoglobin after acidification of the blood to pH values between 6.9 and 7.0 in the plasma was taken as zero, on the assumption of a cell pH at the iso-ionic point of hemoglobin. Constant values of 14 and 9, respectively, were given to the ionic equivalency of the organic phosphates and the osmolarity of the non-diffusible anions in the cells. Given these assumptions, a value of 0.603 gram of plasma water initially and of 0.566 after acidification was calculated. This transfer of water from the plasma to the cells would result in an increase of 6.0 per cent of the corpuscular volume of the cells.

During storage the corpuscular volume increased and the corpuscular hemoglobin concentration correspondingly decreased in bloods stored in the neutral solutions while these indices remained unchanged in bloods collected in the acid medium in which the cells from the outset had assumed an increased volume. It would appear that, in part, the change of the corpuscular volume in the neutral media during storage was due to the lowering of pH with a consequent shift in the distribution of water between cells and plasma, as predicted by the Donnan theory. However, it is evident that other factors are also involved, since the pH in the initially acidified blood continued to decrease without any further change in the corpuscular volume. This behavior will be discussed more fully in a later section dealing with the changes of cations.

Owing to the fact that during storage cations as well as anions permeate the cell membrane, the corpuscular volume of erythrocytes in their own plasma cannot be taken as an indication of alterations occurring in the total osmotic concentration of blood. In this connection it is of interest to compare the volume assumed by erythrocytes of stored blood suspended in 0.9 per cent sodium chloride solution with that observed in their own plasma. From such a comparison a rough indication of the osmotic content of erythrocytes may be expected on condition that in the brief periods of test the cations diffuse only slowly. In column 3 of Table VII are recorded data on the corpuscular volume of erythrocytes of stored blood suspended in 0.9 per cent sodium chloride, expressed as percentage of the simultaneously determined volume in their own plasma. It is apparent that even initially the volume of the erythrocytes in the neutral preservatives, but not in the acid one, exceeded the value measured in their own plasma. After prolonged periods of storage the difference between the 2 measurements increased in all 3 preservatives. The variable initial behavior of erythrocytes in 0.9 per cent sodium chloride does not appear explicable by known osmotic factors.

The explanation nearest at hand for the uniformly increased difference between the volume of erythrocytes in their own plasma and that in 0.9 per cent sodium chloride solution during storage appears to be that, with time, blood becomes increasingly hypertonic with respect to 0.9 per cent sodium chloride solution owing to the breakdown of larger molecules into smaller ones. Three processes occurring during storage which may influence the osmolarity of blood may be cited: (*1*) the accumulation of lactic acid, (*2*) the breakdown of organic phosphates and (*3*) decomposition of glutathione. These processes, detailed consideration of which would occupy too much space, may roughly be estimated to result in an increase of the osmolarity of blood by 5 per cent. This estimate falls below the actually observed change in the corpuscular volume in 0.9 per cent sodium chloride solution during storage, particularly so, when it is considered that some outward diffusion of solutes must take place during the brief exposures to 0.9 per cent sodium chloride solution.

CHEMICAL CHANGES

Plasma pH

The determination of pH was carried out at 37.5° C.; the values recorded in Figure 6 are therefore directly comparable to those customarily measured in biological studies. Under the experimental conditions, where loss of CO_2 owing to exposure to air could not be avoided, the results are of relative value only. The pattern and extent of the changes in pH were obviously related to the rate and amount of lactic acid production during storage, the cessation of the downward shift of pH in citrated blood coinciding with the disappearance of glucose, while in the glucose-enriched media exhaustion of the glycolytic power of the erythrocytes was the limiting factor.

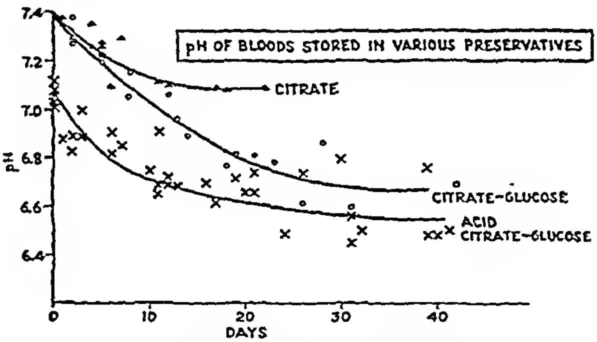

FIG. 6. pH CHANGES DURING STORAGE OF BLOODS STORED IN 3 PRESERVATIVE SOLUTIONS

In general, the pH change during storage in a given blood is dependent on the amount of lactic acid accumulating, on the one hand, and on the type and quantity of buffers present, on the other.

As lactic acid accumulates, CO_2 is lost from the blood in increasing measure, reaching levels as low as 6 meq. per liter after 10 days of storage in glucose-containing media. Among the buffers, hemoglobin is the most important, with phosphates and serum protein following in order. The effect of citrate ion on the ionic balance deserves some comment. The effect of trisodium-citrate on the buffering of the blood may be neglected under the initial conditions of storage, in view of the fact that the pK′ even of the third acid group of citric acid at 5.7 is too far removed from the normal pH of the blood to be of importance. The presence of citric acid in acid citrate-glucose mixture serves more to poise the blood at low pH values from the beginning than to change its buffering qualities, while the addition of tri-sodium citrate in this solution has a negligible effect on either pH or buffering. The great retardation in the rate of fall of pH during storage can be ascribed to 2 factors: (*1*) a decreased rate of glycolysis and with it of lactic acid accumulation and (*2*) the fact that the buffering power of the blood increases as it becomes more acid and is near a maximum at about pH 6.6. Phosphates, present in a concentration of 15 mM. per liter of erythrocytes at this pH, exert their maximum buffering effect, and hemoglobin, close to its iso-ionic point, begins to function as a cation. With lowering of pH the citrate buffering also increases in importance.

Blood sugar, lactic acid, and glycolysis

The sugar concentration of citrated blood, shown in Figure 7A, diminished swiftly, reaching levels of about 10 mgm. per 100 ml. on the eighth day of storage, such levels presumably representing residual nonfermentable reducing substances. The curve of the lactic acid accumulation, portrayed in the same figure, complements that of glucose disappearance. Together the 2 curves demonstrate within the limits of the methods used that glucose was entirely converted into lactic acid, which, once formed, remained unchanged during long periods of storage. In the glucose-enriched media, the glucose decreased by about 200 mgm. from an initial value of about 600 mgm. per 100 ml., the change occurring at a more rapid rate and terminating sooner in neutral, than in acid citrate-glucose. The curves of lactic acid accumulation (Figures 7C and 7E) presenting a complementary picture, with the rate and the final levels higher in neutral, than in acid, citrate-glucose solution.

As an over-all index of their metabolic condition, the glycolytic power of erythrocytes was tested by incubating blood samples for 90 minutes at 37° and measuring the amounts of lactic acid produced and glucose removed during such periods. For the study of the glycolytic faculty of the erythrocytes of citrated blood, which after a few days' storage is depleted of glucose, glucose solution was added before incubation. Inasmuch as no attempt was made to control the pH closely and since the rate of glycolysis is well known to be dependent upon the pH, being much greater at higher pH values, the results of the test have only limited quantitative significance. Individual measurements of rates of 10 mgm. or less per hour are of doubtful significance and attain importance only when evaluated statistically owing to limitations in the accuracy of the methods used. As shown in Figures 7B, 7D, and 7F the glycolytic activity of the erythrocytes ceased earliest in simple citrate and latest in ACD solution. It may also be seen that the erythrocytes had a higher initial glycolysis rate in the 2 neutral media than in the acid one.

The significance of glycolysis in the functioning of erythrocytes lies in the fact that glycolysis is the most important energy-yielding process occurring in the cells. Their oxygen consumption normally is minute, accounting for less than 10 per cent of the energy yielded by glycolysis. The weakness of the aerobic metabolism of erythrocytes is further illustrated by the virtual absence of the Pasteur effect, as indicated by the fact that glycolysis proceeds at essentially the same rate in oxygen as under anaerobic conditions (42). Before accepting the significance of the glycolytic process in the functioning of erythrocytes, one may consider the question of their metabolic energy requirements. These cells, after maturation from the reticulocyte stage, do not appear to undergo further development nor do they multiply. Their most impressive attribute is the biconcave form, which is maintained under a variety of conditions throughout their life. There has been considerable speculation regarding the mechanism for the maintenance of the characteristic form of erythrocytes (43). Generally 2 basic hypotheses have been advanced: (*1*) the existence of a rigid structure and

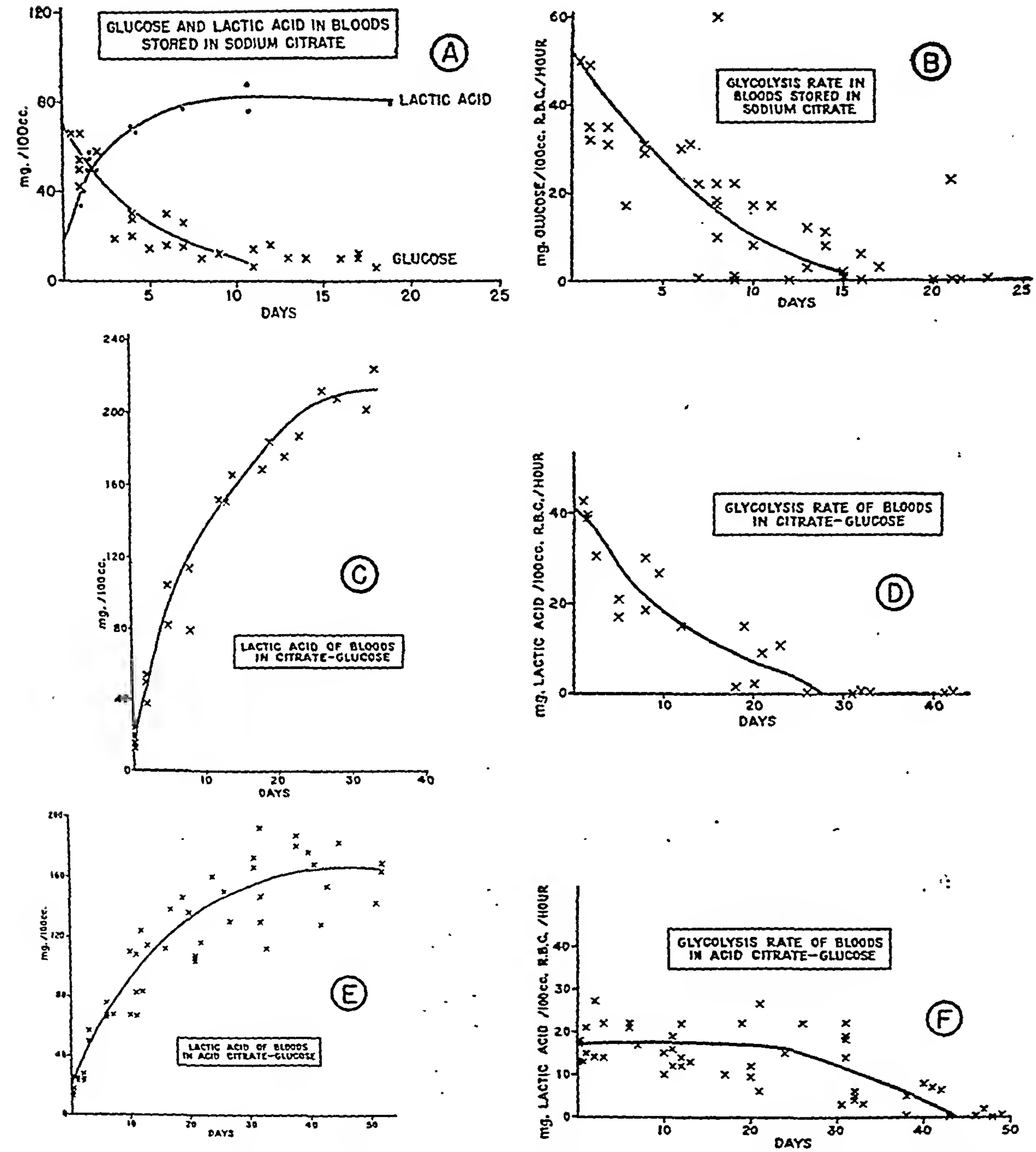

FIG. 7. CHANGES IN GLUCOSE, LACTIC ACID AND GLYCOLYSIS RATE OF BLOODS STORED IN 3 PRESERVATIVE SOLUTIONS

(2) a dynamic balance of forces resulting in constancy of the cellular shape. If, under most conditions, the erythrocytes do not possess a rigid structure, as is indicated by considerable evidence, the conclusion is inescapable that continuous expenditure of energy is required for the preservation of the biconcave form. An attractive hypothesis would then be that in part the function of the glycolytic process is to yield the energy required for the maintenance of the cellular form.[3] Ac-

[3] Assuming a surface tension of 60 dynes per cm., the energy required by a single cell would be in the order of

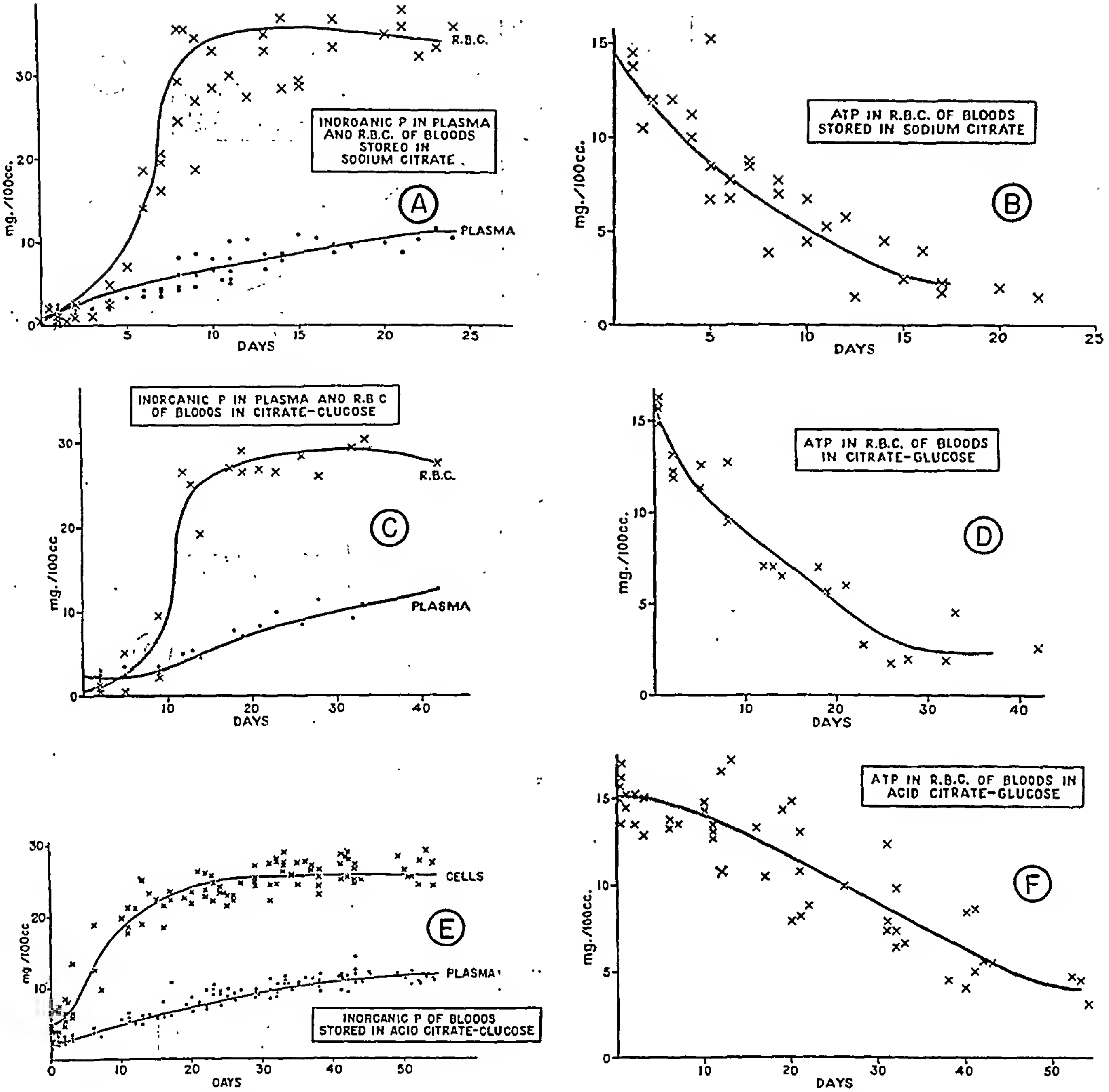

FIG. 8. CHANGES IN THE INORGANIC PHOSPHORUS DISTRIBUTION AND THE ADENOSINE TRIPHOSPHATE CONTENT OF RED CELLS DURING STORAGE OF BLOOD IN 3 PRESERVATIVE SOLUTIONS

cepting this premise, the similarity of patterns of dimensional and chemical changes and their close correspondence would reflect a causal relationship.

10^{-5} erg. Assuming a corpuscular volume of 90 micra^3, 100 ml. of cells would contain about 10^{12} erythrocytes, with an energy requirement of 10^7 ergs, that is about 0.25 cal. This would correspond to the energy yielded by glycolysis of about 2 mgm. of glucose per 100 ml. of cells. If the surface tension of the cell and plasma is less, as is indicated by some studies, the energy requirement would be correspondingly decreased.

Acid-soluble phosphorus compounds

Inorganic phosphorus in cells and plasma: The inorganic phosphorus in the red cells is shown in Figures 8A, 8C, and 8E. In the neutral media, it rose rapidly following a period of delay while in the acid medium, it rose from the start but at a lower rate. The inorganic phosphorus of the plasma in all media rose at an approximately linear rate.

Adenosine triphosphate: The fate of adenosine

triphosphate (ATP) during storage was of particular interest since this compound is an important co-enzyme of glycolysis. Data for this compound, calculated from the values for the easily-hydrolyzable phosphorus, are presented in Figures 8B, 8D, and 8F. It may be seen that it decreased most slowly in the blood stored in ACD solution.

Adenosine triphosphate was determined more specifically in several bloods as the easily-hydrolyzable phosphorus in the mercury precipitate of the trichloracetic acid filtrate. The results, not presented here in detail, indicated for citrated blood an initial concentration of about 12 mgm. per 100 ml. of cells diminishing to values below 2 mgm. after about 10 days of storage. The adenosine triphosphate changes in the glucose-enriched media bore the same relation to those in citrated blood as were given by the cruder method described previously. Studies were also carried out on the fate of the other components of the adenosine triphosphate molecule during storage besides that of the 2 easily hydrolyzable phosphate groups. Adenine, hypoxanthine, and purine nitrogen were determined in the whole blood and in fractions comprising nucleotides on the one hand and nucleosides plus free purines on the other. By following the pentose content of the mercury precipitate of the trichloroacetic acid extract, which contains all the nucleotide and a considerable proportion of the nucleoside, a rough measure of pentose combined with purine was obtained. The results, to be presented in detail elsewhere, indicated that the several parts of the adenosine triphosphate molecule underwent changes during storage according to differing patterns and at varying rates. The most rapid, and for the glycolytic process most critical change, was the loss of the 2 easily hydrolyzable phosphate groups. As a consequence, free adenylic acid accumulated. Adenine was deaminated to hypoxanthine, the conversion reaching as much as 80 per cent. The deamination seemed to be accompanied in each case by the loss of the third phosphate group, leading to the accumulation of hypoxanthine riboside after prolonged periods of storage.

Organic acid-soluble phosphorus: The changes in the organic acid-soluble phosphorus of the cells, which consists for the most part of a mixture of adenosine triphosphate and diphosphoglyceric acid, mirrored to a large extent those of inorganic phos-

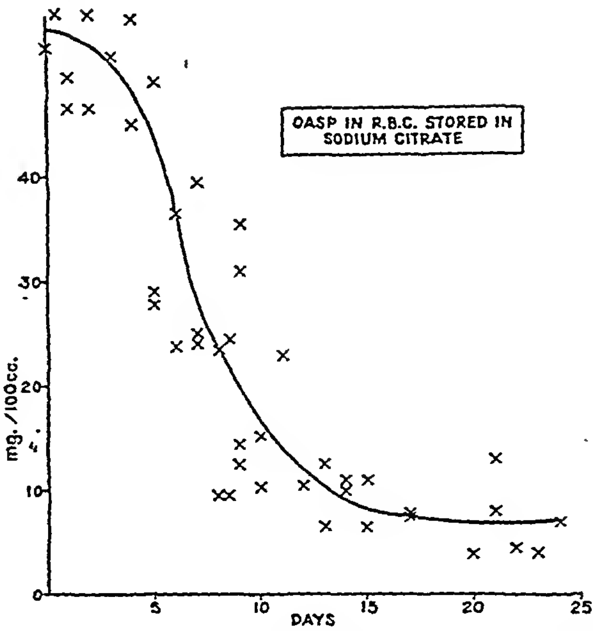

Fig. 9. Organic Acid Soluble Phosphorus in Blood
Stored in Sodium Citrate

phorus as shown for citrated blood in Figure 9

The total acid-soluble phosphorus content of all bloods remained unchanged even after the most extended storage periods (70 days), indicating that the phospholipids, which in mammalian bloods almost entirely account for the acid-insoluble phosphorus fraction, were not hydrolyzed under such conditions.

A discussion of the changes in the phosphorus distribution of blood during storage necessitates a sketchy review of the changes occurring during glycolysis at 37° C., and a brief consideration of the role of the phosphorus compounds in the intermediate reactions of the glycolytic process (44). The glycolytic system of the erythrocytes is distinguished by great sensitivity to changes of pH, the over-all rate of glycolysis increasing with elevation of pH. If glycolysis is allowed to proceed at pH values above 7.3, the inorganic phosphorus, following a brief period during which the level may decrease, steadily increases, at the expense primarily of adenosine triphosphate and later also of diphosphoglyceric acid. During incubation at pH values below 7.3, the inorganic phosphorus rises from the start at a rapid rate, while the adenosine triphosphate is maintained at a high level and the diphosphoglyceric acid diminishes swiftly (45). The resemblance beween the changes occurring

at 37° C. and those taking place in bloods during storage at low temperatures is obvious: In bloods collected in neutral media, a short period of unchanged or decreased levels of inorganic phosphorus is followed by a breakdown of both adenosine triphosphate and diphosphoglyceric acid, as judged by the data described earlier; in bloods stored in acid citrate-glucose, an immediate rise of the inorganic phosphorus goes hand in hand with the maintenance of high levels of adenosine triphosphate for considerable periods of time. Decreasing values of the total organic acid-soluble phosphorus were, by inference, accounted for by changes in the diphosphoglyceric acid.

In the light of modern concepts of the glycolytic process (45, 46, 47) the changes in the glycolytic rate and in the distribution of phosphorus compounds at varying pH values may be interpreted as follows: At elevated pH values glycolysis proceeds at a rapid rate, while the level of adenosine triphosphate tends to decrease; this indicates that under such circumstances the rate of esterification of glucose by adenosine triphosphate proceeds at a more rapid rate than does the rephosphorylation of adenylic acid. In acidified blood, where the glycolytic rate may be only one-third as great as normal the adenosine triphosphate is maintained at a higher level, indicating that rephosphorylation of adenylic acid keeps pace with the esterification of glucose. This balance is in part ascribable to the decreased over-all rate of turnover; in addition, under such circumstances diphosphoglyceric acid in decomposing furnishes an additional source of phosphorus for the re-synthesis of adenosine triphosphate. Since adenosine triphosphate appears less susceptible to irreversible deaminating attack than adenylic acid, circumstances leading to minimum concentrations of adenylic acid, such as prevail in acidified blood, tend to slow the rate of breakdown of the essential nucleotides.

From inspection of the data it becomes evident that the glycolytic power of the erythrocytes was still present to some extent at a time when most of the organic acid-soluble phosphorus had decomposed. The lack of correlation between the 2 indices suggests that only a small component of the organic acid-soluble phosphorus, perhaps the adenosine triphosphate, or a portion thereof, constitutes the limiting factor in the glycolytic process.

On the basis of the data presented one may venture the hypothesis that the maintenance of the functional state of the cell and of its various characteristics, dimensional, osmotic, and chemical, is dependent upon the glycolytic process which in turn is dependent on the intact functioning of the enzyme system concerned with it; adenosine triphosphate, or a compound determining its concentration, is of critical importance for the preservation of the glycolytic power of the erythrocytes. It may be surmised that only cells which by virtue of a functioning enzyme structure have the ability to recover their normal physical and chemical structure survive *in vivo*.

Potassium and sodium

The changes in the potassium content of serum and cells are portrayed in Figures 10A, 10C, and 10E. It can be seen that the potassium content of plasma of citrated blood increased in the beginning at the rate of about 2.0 meq. per liter per day and more slowly after longer periods, reaching a value halfway towards diffusion equilibrium in about 8 days. Half-diffusion-equilibrium values were reached after 12 days in citrate-glucose and after about 23 days in acid citrate-glucose. The changes of potassium in the cells in each case mirrored faithfully those occurring in plasma.

The sodium in plasma and cells, as shown in Figures 10B, D, and F, underwent in all solutions a change reciprocal to that of the potassium, but at a slower rate. A comparison of the 3 preservatives with regard to sodium leads to essentially the same conclusion as described above for potassium.

It is of interest to examine to what extent the exchange of potassium and sodium between cells and plasma follows the law of simple diffusion, particularly so, in view of the possibility that the normally existing inequality of the ionic concentrations between cells and plasma is maintained by metabolic processes. It would appear that the processes of ionic exchange, except for the first few days, may be described on the assumption of simple diffusion, with rates decreasing in order in citrate, neutral, and acid citrate-glucose solutions.

The changes in the ionic composition of blood during storage have obvious implications with regard to the general theory of permeability of the erythrocyte membrane. On the one hand, it appears that sodium and potassium exchange across the cellular membrane during storage (as indeed

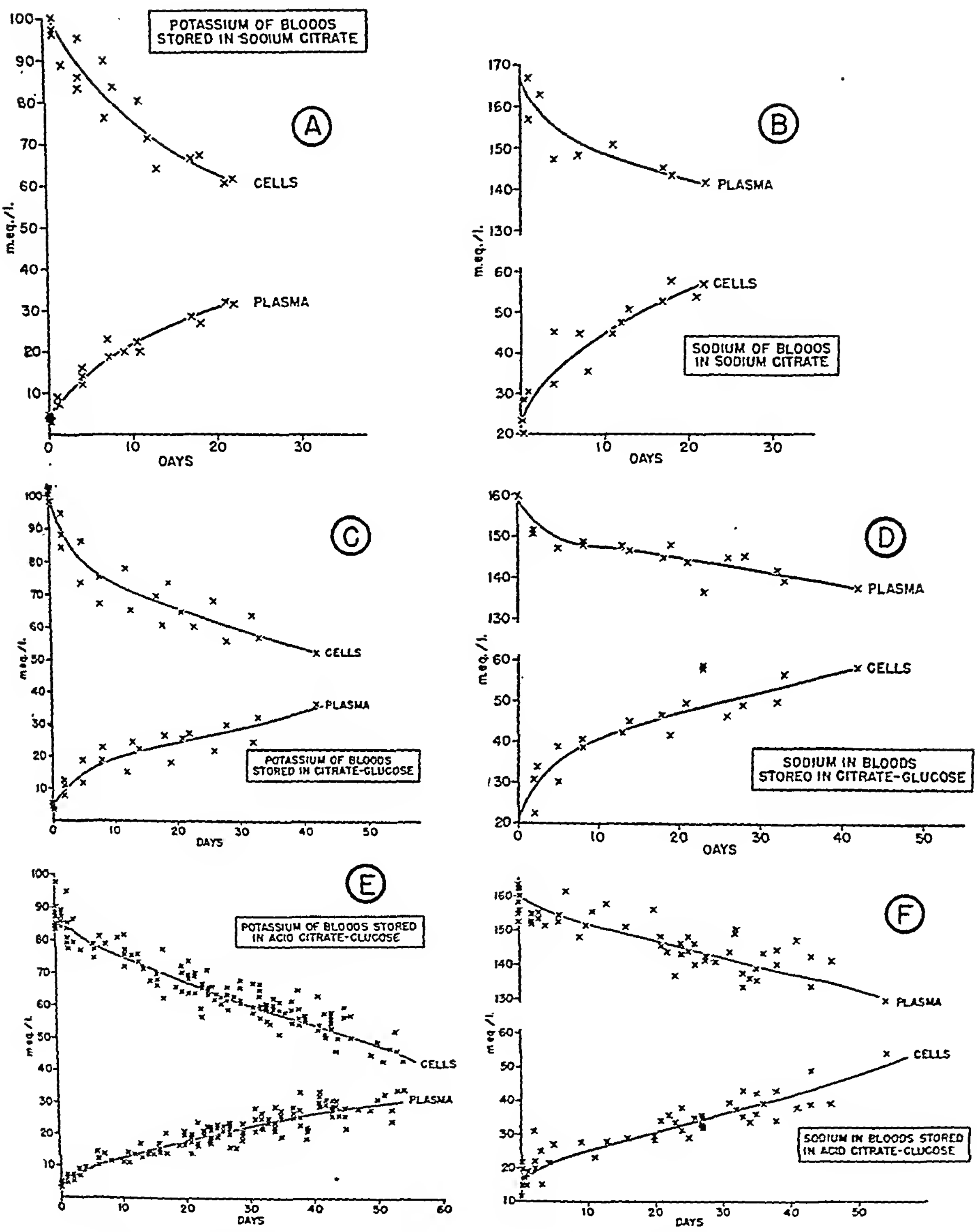

Fig. 10. Changes in Distribution of Potassium and Sodium between Plasma and Cells of Bloods Stored in 3 Preservative Solutions

in vivo, as shown by the radioactive tracer technique [48 to 50]); on the other hand, there exists considerable evidence which indicates that the exchange of water and anions between cells and plasma may be described by the Donnan theory on the postulate of non-permeability of the erythrocyte membrane to cations (40, 51, 52). In order to conciliate this apparent contradiction, it has been proposed that the cations exchange across the cellular membrane not by way of simple diffusion, but rather by means of a metabolic process. Such a hypothesis would also account for the unequal distribution of potassium and sodium between cells and plasma. The decrease of cellular potassium during storage may then be thought to be due to unfavorable conditions for the specific metabolic process concerned with the transfer of potassium so that the normally existing steady state is no longer maintained. According to another explanation which is not necessarily in conflict with the one just mentioned, the non-diffusibility of the cations may be due to some form of binding of the cations. However, existing data indicate that at least 90 per cent of the cell water is "free," that is, osmotically active, and that the degree of osmotic activity of cations in the cells does not differ markedly from that in the plasma. It is still conceivable that there exists within the cells a restraining influence which, while preventing the free outward diffusion of cations, interferes little with their osmotic activity. This hypothesis gains interest in line with observations to be presented in the following paragraphs.

In view of the intimate connection between the electrolyte concentration and the water content of the cells, it is of interest to examine the changes during storage in the total concentration of cations. In Table VIII are presented the mean values for the sum of sodium and potassium, taken to represent the total cations, observed on blood in acid citrate-glucose solution initially and after periods of storage of 20 to 40 days. The concentrations in cells and plasma are recorded in meq. per liter of each, and per kgm. of water. On either basis, it it evident that the cells in acid citrate-glucose solution actually lost cations during storage. This loss apparently occurred in a manner that left the water distribution, and with it the cellular volume, unchanged. The amount of cation transferred from cells to plasma agrees well with the value calculated from the ionic equivalency of hemoglobin when the water content of the cells remains constant. This is also shown in the table. The behavior of the erythrocytes suggests that their corpuscular volume in acidified blood is maintained by a graded transfer of cation in apparent proportionality to the increased ionization of hemoglobin as a cation. To explain the unexpected behavior the following hypothesis may be tentatively adduced: assuming that the apparent non-diffusibility of cations across the cellular membrane is caused by some restricting interaction, perhaps a molecular force existing between the cations and other components within the cell, one may reason

TABLE VIII

Distribution of cations between cells and plasma during storage of blood in acid citrate-glucose solution

The results are expressed as the mean, together with the standard error of the mean.

Period of storage		Cells Na+K	Plasma Na+K	Plasma pH
days				
0	*meq. per liter*	108.8 ±3.8	160.2 ±3.1	6.95
	*meq. per kgm. water**	148	168	
	Calculated value** *meq. per liter*	112	157	
20–30	*meq. per liter*	95.3 ±1.1	167.7 ±1.1	6.50
	*meq. per kgm. water**	130	176	
	Calculated value** *meq. per liter*	100	164	
	Change during storage:			
	meq. per liter	−13.5 ±4.0	+7.5 ±3.3	−0.35
	*meq. per kgm. water**	−18	+8	
	*p****	0.001	<0.05	
	Calculated value** *meq. per liter*	−12	+7	

* A constant value of 0.743 gram per ml. was calculated for the cell water by means of the formula

$$H_2O_c = 94.53 - 0.704\ Hb_c$$

where Hb_c indicates the concentration of hemoglobin in gram per 100 ml. of cells, on the basis of a measured hemoglobin concentration of 30.0 grams per 100 ml. The plasma water was assumed to be 0.95 gram per ml.

** These values were calculated by means of equations and assumptions described earlier, on the basic premise that the water distribution between cells and plasma remained constant during storage and that the only variable was the ionic equivalency of hemoglobin, for which the values given by Adair (41) were used. For the calculation of the initial values of cell and plasma water it was assumed that the blood in the beginning was at a pH close to the iso-ionic point of hemoglobin. As may be seen, the values calculated on this rough approximation were in fairly good agreement with the observed values. Given an acidification to a pH in the plasma of 6.50 during storage, the changes in the distribution of cations in plasma and cells were calculated with the same values for the serum and cell water as assumed initially.

*** p = the percentage chance that a deviation as great or greater than that observed would arise by chance alone; any value of p 0.05 or less is usually accepted as indicating a significant difference.

that the hemoglobin cation competes with the monovalent cations, displacing them from positions previously occupied, perhaps by virtue of its greater size. The displaced monovalent cations, no longer restrained, would then freely leave the erythrocyte. Since diffusible anions would enter the cell in direct proportion to the cationic ionization of hemoglobin and an equal proportion of cations becoming diffusible would leave the cell constant osmolarity and therefore constant water content would be maintained. According to this hypothesis, therefore, erythrocytes would tend to maintain constant volume below the iso-ionic point of hemoglobin, by means of loss of cations in direct proportion to the ionization of hemoglobin as a cation.

STATISTICAL COMPARISON OF 5 INDICES OF BLOOD PRESERVATION

In Table IX are presented data based on 96 blood samples on the degree of association among the 5 indices of blood stored in 3 preservative solutions. The table contains values for the regression equations and correlation coefficients between percentage hemolysis in 0.6 per cent NaCl solution, which (as the most precise measurement) was chosen as the basis of comparison on the one hand and the 4 other indices on the other. It may be seen that for each of the preservative solutions close association existed between the alterations of hemolysis in 0.6 per cent NaCl solution and the changes of any of the 4 other indices, the only exception being glycolysis of blood in simple citrate, the values for which were rather variable. An inspection of the data indicates that the absolute values of the regression coefficients were of similar magnitude in all 3 preservatives. Similarity in the relative rates of change of all 5 indices in the 3 preservatives is indicated by the highly significant values for the regression and correlation coefficients for the pooled values. In Table X are recorded for the purpose of close comparison the differences among the several regression coefficients for the 3 preservative solutions. It may be seen that except for 2 values, the differences between the several regression coefficients were of small magnitude. When the individual regression coefficients in each of the 3 preservative solutions were compared with the pooled estimate, no statistically significant difference was apparent in any instance.

The similarity of the regression functions with change in hemolysis for the dimensional, metabolic, and chemical characteristics of the red cell in the 3 solutions of widely differing preservative qualities indicates that the several indices displayed similar relationships among each other despite great variations, up to 4-fold, in the rate of change with time. The data as a whole represent indirect evidence of close interdependence of physical, osmotic, and chemical properties of the red cell.

DISCUSSION

From a review of the composite findings presented, it appears that a number of apparently unrelated properties of the erythrocyte undergo

TABLE IX

Relationship between hemolysis in 0.6 per cent NaCl solution and 4 other indices of bloods stored in 3 preservative solutions
Regression equations and correlation coefficients

Solution	Thickness vs. hemolysis		Adenosine triphosphate vs. hemolysis		Glycolysis vs. hemolysis		Potassium vs. hemolysis	
	Regression equation	Correlation coefficient	Regression equation	Correlation coefficient	Regression equation	Correlation coefficient	Regression equation	Correlation coefficient
Citrate	$-0.011+0.0187x$ ±0.0014	+0.946	$10.8-0.123x$ ±0.017	−0.866	$23.7-0.247x$ ±0.100	−0.484	$11.2+0.272x$ ±0.054	+0.824
Citrate-glucose	$0.041+0.0169x$ ±0.0015	+0.914	$11.6-0.145x$ ±0.017	−0.914	$24.3-0.390x$ ±0.082	−0.767	$16.1+0.199x$ ±0.049	+0.708
Acid citrate-glucose	$0.030+0.0145x$ ±0.0007	+0.960	$13.2-0.142x$ ±0.017	−0.767	$17.5-0.283x$ ±0.039	−0.753	$9.7+0.345x$ ±0.028	+0.870
Pooled value of 3 preservatives	$0.014+0.0168x$ ±0.0006	+0.922	$12.6-0.147x$ ±0.004	−0.755	$20.1-0.281x$ ±0.042	−0.539	$11.5+0.285x$ ±0.023	+0.732

TABLE X

Relationship between hemolysis in 0.6 per cent NaCl solution and 4 other indices of bloods stored in 3 preservative solutions

Differences between regression coefficients and estimates of their significance

Comparison	Thickness vs. hemolysis	Adenosine triphosphate vs. hemolysis	Glycolysis vs. hemolysis	Potassium vs. hemolysis
Citrate vs. citrate-glucose	+0.0018	+0.022	+0.143	+0.073
	±0.0020	±0.024	±0.133	±0.073
p*	0.4	0.4	0.3	0.3
Citrate vs. acid citrate-glucose	+0.0042	+0.003	−0.036	−0.073
	±0.0015	±0.024	±0.112	±0.061
p*	0.01	0.9	0.8	0.3
Citrate-glucose vs. acid citrate-glucose	+0.0024	−0.019	+0.107	−0.146
	±0.0016	±0.024	±0.091	±0.057
p*	0.15	0.5	0.3	0.02

* p = percentage chance that a deviation as great or greater than that observed would arise by chance alone; any value of p 0.05 or less is usually accepted as indicating a significant difference. The bold-faced figures indicate significant differences.

changes in a characteristic manner during storage. These alterations follow a similar pattern in each of the 3 preservatives studied. A brief listing of the cellular characteristics which changed in a critical and characteristic manner during storage may be useful at this point. They were: (*1*) hemolysis in several concentrations of NaCl solution from 0.9 per cent down, (*2*) dimensional changes of the erythrocyte, *i.e.*, in diameter, thickness, and surface area, (*3*) glycolyzing power, (*4*) adenosine triphosphate content, and (*5*) change in the distribution of potassium. Not all physical or chemical properties of the cell followed this pattern; for instance, the corpuscular volume appears to be determined by the osmotic and ionic relationships existing between cells and serum rather than by the functional state of the cell. The liberation of inorganic phosphorus and alterations of the glutathione content belong also in the group of cellular properties, which did not change in a manner which can be correlated with the preservation of the red cell. Such differences in behavior suggest that, while some of the cellular properties are intimately related to the metabolic activity of the cell and reflect its functional state, others do not bear such a relationship. To some extent, the division may be artificial and dependent on the scale and method of measurement adopted. This is illustrated by the conflicting experiences with the use of osmotic hemolysis as an index of blood preservation.

Comparison of in vitro *and* in vivo *tests*

The practical importance of *in vitro* data hinges on their close correlation to the *in vivo* survival of erythrocytes. In order to compare the results reported here with the *in vivo* behavior of stored blood, the data on the percentage survival of erythrocytes 7 days after transfusion obtained (6) by a differential agglutination method have been charted in Figure 11. The preservatives tested by

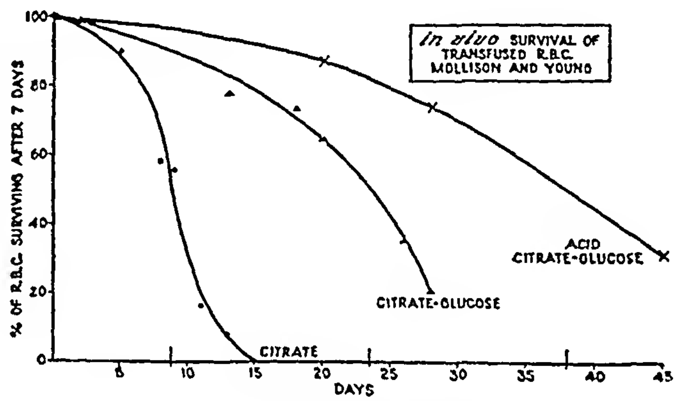

FIG. 11. *In vivo* SURVIVAL OF ERYTHROCYTES OF BLOODS STORED IN 3 PRESERVATIVE SOLUTIONS ACCORDING TO DATA OF MOLLISON AND YOUNG (6)

the British workers, except for the use of a higher concentration of citrate in the neutral media, were closely comparable to those studied here. Similar results have been obtained by the use of radioactive iron for the direct measurement of the survival of the erythrocytes (Gibson, Ross). The close correspondence between *in vitro* and *in vivo* results indicates the practical usefulness of *in vitro* methods and supports the initial hypothesis of close interrelation between many properties of the red cell.

The principal applications of *in vitro* methods appear to be two: first, as preliminary procedures for the screening of preservative solutions before they are submitted to definitive testing by established *in vivo* methods. Their rapidity, sensitivity and accuracy, make them well suited for this purpose. As to accuracy, one advantage lies in the fact that only the variability of the donor comes into play, whereas *in vivo* testing is affected by the cumulative variabilities of donor and recipient.

Second, *in vitro* methods have their widest field of use in the investigation of the mechanism of deteriorative processes of the cell and constitute therefore the basis for rational elaboration of improved preservative techniques. For instance, they already appear to furnish some understanding of the apparent advantage of the addition of glucose and of acid to blood. Their failure under certain conditions to reflect the functional state of the cell is perhaps more an index of the deficiencies of our understanding than a general argument against their validity. The employment of a series of indices for the description of the preservation of the red cell should help to guard against the danger of reliance on any single index, which under certain experimental conditions may prove to be a fallacious guide.

Practical considerations

While the length of preservation appears of paramount importance in the selection of preservative solutions, other factors merit attention in the consideration of the practical usefulness of any preservative. Among these may be mentioned: (*1*) ease of sterilization of the preservative solution, (*2*) extent of dilution of the blood, (*3*) suitability for the subsequent preparation of fluid or dried plasma, and (*4*) possible deleterious effects of ingredients of the preservative solutions proposed. For instance, with respect to the first point, neutral citrate-glucose solution, which requires a special technique to prevent caramelization of glucose, is at a disadvantage compared with simple citrate solution. ACD solution on the other hand may be sterilized as simply as citrate solution alone. In view of the superiority of this solution with respect to the longevity of red cells, the subsequent discussion will be limited to consideration of its merits and demerits. Its use entails a dilution of blood to the extent of 25 per cent, so that the concentration of protein in the plasma is reduced to about 5.1 grams per 100 ml., assuming an initial concentration of 7 grams per 100 ml. and taking into account the swelling of the erythrocytes. The protein concentration of plasma of blood in simple citrate, calculated on the same assumption, is about 5.8 grams per 100 ml. With respect to the preparation of dried plasma, some increase in drying time may be expected owing to the presence of glucose in acid citrate-glucose solution. The possibility of deleterious effects of the acidification of blood may be dismissed on theoretical grounds as well as on the basis of practical experience. Injection of an organic acid, which is easily metabolized in the body with the production of CO_2, in amounts such as are added to blood, may be confidently expected not to produce an appreciable effect on the ionic equilibrium of the blood of the recipient. Direct experiments on this point have been carried out (6). A comparable condition exists in any stored blood, where the amounts of lactic acid accumulated are about as much as the amounts of citric acid added. In summary one may therefore say that, while acid citrate-glucose solution produces a somewhat more dilute plasma and may give rise to minor difficulties in the production of dried plasma, its greatly superior preservative qualities make it the preservative solution of choice.

SUMMARY

On the basic premise that the erythrocyte represents a functional unit, with closely interdependent physical and chemical properties, which in turn are related to its viability, a study of dimensional, osmotic, and chemical changes of blood stored in 3 preservative solutions was undertaken. The preservatives studied were: (*1*) simple citrate solution, (*2*) a neutral, and (*3*) an acid citrate-glucose (ACD) solution. The results of this investigation may be summarized as follows:

(*1*) During storage red cells undergo a change in their dimensions in a characteristic and critical manner; their thickness increases, their diameter decreases, and their apparent surface area is reduced. These alterations are interpreted as manifestations of progressive deterioration of the cell. They progress most rapidly in blood in simple citrate, and most slowly in blood in acid citrate-glucose solution. Changes in the corpuscular volume appear to be determined by physico-chemical relationships and not to be related to the functional state of the cell.

(*2*) Osmotic hemolysis of erythrocytes becomes similarly altered in a progressive manner during storage, at a rapid rate in simple citrate and most slowly in acid citrate-glucose solution.

(*3*) The plasma pH decreases to a value of 7.1

in simple citrate solution, and to a value below 6.6 in the glucose-enriched media in direct relation to the amount of lactic acid accumulated.

(*4*) The ability of the red cells to glycolyze is lost first in simple citrate solution and is preserved longest in acid citrate-glucose solution.

(*5*) The inorganic phosphorus increases sharply in the red cells, with corresponding decrease of the organic acid-soluble phosphorus, but changes in a more gradual manner in the plasma. Adenosine triphosphate, a co-enzyme of blood glycolysis, is preserved considerably longer in blood in ACD solution than in the 2 neutral media. Maintenance of adenosine triphosphate appears of importance for the preservation of the metabolic function of erythrocytes.

(*6*) Decreases of potassium and increases of sodium in erythrocytes reported by others have been confirmed. During storage the total cation content of erythrocytes decreases. This fact and the constancy of the corpuscular volume of erythrocytes in ACD solution during storage are interpreted as indicating loss of cations on the acid side of the iso-ionic point of hemoglobin, in direct proportion to the dissociation of hemoglobin as a cation, with the osmolarity of the cells remaining constant.

Judged by various indices the state of erythrocytes of citrated blood after 8 days is approximately equivalent to their state in neutral citrate-glucose after 18 days and in ACD solution after 33 days.

It would appear that *in vitro* testing has its place in the study of preservation of erythrocytes (*1*) as a useful preliminary procedure for the screening of preservative solutions before these submitted to definitive testing by direct measurement of their survival *in vivo*, and (*2*) as a useful tool in the development of improved methods of blood preservation on a rational basis.

ACD solution is recommended for practical use.

ACKNOWLEDGMENT

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BIBLIOGRAPHY

1. Rous, P., and Turner, J. R., The preservation of living red blood cells *in vitro*. I. Methods of preservation. II. The transfusion of kept cells. J. Exper. Med., 1916, 23, 219.
2. Maizels, M., and Whittaker, N., Effects of storage on human erythrocytes. Lancet, 1940, 1, 113.
3. Wilbrandt, W., Ueber Konservierung menschlichen Blutes. Schweiz. Med. Wchnschr., 1940, 70, 731.
4. Mollison, P. L., and Young, I. M., *In vivo* survival in the human subject of transfused erythrocytes after storage in various preservative solutions. Quart. J. Exper. Physiol., 1942, 31, 359.
5. Bushby, S. R. M., Kekwick, A., Marriott, H. L., and Whitby, L. E. H., Survival of stored red cells after transfusion. Lancet, 1940, 2, 414.
6. Loutit, J. F., Mollison, P. L., and Young, I. M., Citric acid-sodium citrate-glucose mixtures for blood storage. Quart. J. Exper. Physiol., 1943, 32, 183.
7. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. I. Various factors influencing hemolysis. J. A. M. A., 1940, 114, 850.
8. Alsever, J. B., and Ainslie, R. B., A new method for the preparation of diluted blood plasma and the operation of a complete transfusion service. New York State J. Med., 1941, 41, 126.
9. Muether, R. O., and Andrews, K. R., Studies on stored blood. II. Effect of storage on human blood. Am. J. Clin. Path., 1941, 11, 314.
10. Mollison, P. L., and Young, I. M., Failure of *in vitro* tests as a guide to the value of stored blood. Brit. M. J., 1941, 2, 797.
11. Maizels, M., Phosphate, base and hemolysis in stored blood. Quart. J. Exper. Physiol., 1943, 32, 143.
12. Belk, W. P., Henry, N. W., and Rosenstein, F., Observations on human blood stored at 4 to 6 degrees centigrade. Am. J. M. Sc., 1939, 198, 631.
13. Belk, W. P., and Rosenstein, F., Effect of glucose in preservation of citrated human blood stored at 4° to 6° centigrade. Am. J. M. Sc., 1941, 201, 841.
14. Strumia, M. M., Symposium on new trends in surgery. The fate of transfused refrigerated blood and the problem of blood banks. S. Clin. North America, 1942, 22, 1693.
15. Bagdassarov, A., Le problème de la transfusion du sang conservé. Sang, 1937, 11, 466.
16. Dubash, J., Clegg, O., and Vaughan, J., Changes occurring in blood stored in different preservatives. Brit. M. J., 1940, 2, 482.
17. De Gowin, E. L., Harris, J. E., Bell, J., and Hardin,

R. C., Osmotic changes in erythrocytes of human blood during storage. Proc. Soc. Exper. Biol. and Med., 1942, 49, 484.

18. Crosbie, A., and Scarborough, H., Studies on stored blood. Further observations on the effect of storage on erythrocytes. Edinburgh M. J., 1942, 49, 40.
19. Drew, C. R., Edsall, K., and Scudder, J., Studies in blood preservation. Fate of cellular elements in relation to potassium diffusion. J. Lab. and Clin. Med., 1939, 25, 240.
20. De Gowin, E. L., Harris, J. E., and Plass, E. D., Changes in human blood preserved for transfusion. Proc. Soc. Exper. Biol. and Med., 1939, 40, 126.
21. Conway, E. J., Apparatus for micro-determination of certain volatile substances. IV. The blood ammonia, with observations on normal human blood. Biochem. J., 1935, 29, 2755.
22. Conway, E. J., and Cooke, R., Blood ammonia. Biochem. J., 1939, 33, 457.
23. Smith, M. E., Tuthill, E., Drew, C. R., and Scudder, J., Studies in blood preservation. Some effects of carbon dioxide. J. Biol. Chem., 1940, 133, 499.
24. Dulière, W. L., La teneur en potassium et l'age d'un sérum. Compt. rend. Soc. de biol., 1931, 107, 261.
25. Jeanneney, G., and Servantie, L., Valeur du dosage de potassium comme test d'alteration du sang conservé. Compt. rend. Soc. de biol., 1938, 129, 1189.
26. De Gowin, L. E., Harris, J. E., and Plass, E. D., Studies on preserved human blood. II. Diffusion of potassuim from the erythrocytes during storage. J. A. M. A., 1940, 114, 855.
27. Downman, C. B. B., Oliver, J. O., and Young, I. M., Partition of potassium in stored blood. Brit. M. J., 1940, 1, 559.
28. Maizels, M., and Paterson, J. H., Survival of stored blood after transfusion. Lancet, 1940, 2, 417.
29. Aylward, F. X., Mainwaring, B. R. S., and Wilkinson, J. F., Effects of some preservatives on stored blood. Lancet, 1940, 1, 685.
30. Maizels, M., Preservation of organic phosphorus compounds in stored blood by glucose. Lancet, 1941, 1, 722.
31. Denstedt, O. F., Osborne, D. E., Roche, M. N., and Stansfield, H., Problems in the preservation of blood. Canad. M. A. J., 1941, 44, 448.
32. Loutit, J. F., and Mollison, P. L., Advantages of a disodium-citrate-glucose mixture as a preservative. Brit. M. J., 1943, 2, 744.
33. Maizels, M., Observations on stored blood. Report to M.R.C. Blood Transfusion Research Committee, January, 1943.
34. Guest, G. M., and Wing, M., Osmometric behavior of normal human erythrocytes. J. Clin. Invest., 1942, 21, 257.
35. Rapoport, S., and Guest, G. M., The effect of salicylates on the electrolyte structure of the blood plasma. I. Respiratory alkalosis in monkeys and dogs after sodium and methyl salicylate; the influence of hypnotic drugs and of sodium bicarbonate on salicylate poisoning. J. Clin. Invest., 1945, 24, 759.
36. Nelson, N., A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 1944, 153, 375.
37. Friedemann, T. E., Cotonio, M., and Shaffer, P. A., The determination of lactic acid. J. Biol. Chem., 1927, 73, 335.
38. Dacie, J. V., and Vaughan, J. M., Fragility of red blood cells; its measurement and significance. J. Path. and Bact., 1938, 46, 341.
39. Jacobs, M. H., and Parpart, A. K., Osmotic properties of the erythrocyte. II. The influence of pH, temperature, and oxygen tension on hemolysis by hypotonic solutions. Biol. Bull., 1931, 60, 95.
40. Van Slyke, D. D., Wu, H., and McLean, F. C., Studies of gas and electrolyte equilibria in the blood. V. Factors controlling the electrolyte and water distribution in the blood. J. Biol. Chem., 1923, 56, 765.
41. Adair, G. S., Hemoglobin system. Relation of hemoglobin and bases. J. Biol. Chem., 1925, 63, 517.
42. Lundsgaard, E., Die Glykolyse. Ergeb. d. Enzymforsch., 1933, 2, 179.
43. Ponder, E., The Mammalian Red Cell and the Properties of Haemolytic Systems. Berlin, Gebr. Borntraeger, 1934.
44. Guest, G. M., and Rapoport, S., Organic acid-soluble phosphorus compounds of the blood. Physiol. Rev., 1941, 21, 410.
45. Rapoport, S., and Guest, G. M., Decomposition of diphosphoglycerate in acidified blood: its relationship to reactions of the glycolytic cycle. J. Biol. Chem., 1939, 129, 781.
46. Hevesy, G., and Aten, A. H. W., Interaction of plasma phosphate with the phosphorus compounds present in the corpuscles. Kgl. Danske Videnskab. Sleskab. Biol. Medd., 1939, 14, no. 5.
47. Hevesy, G., and Hahn, L., Rate of renewal of the acid-soluble organic phosphorus compounds in the organs and the blood of the rabbit. Kgl. Danske Videnskab. Sleskab. Biol. Medd., 1940, 15, no. 7.
48. Dean, R. B., Noonan, T. R., Haege, L., and Fenn, W. O., Permeability of erythrocytes to radioactive potassium. J. Gen. Physiol., 1941, 24, 353.
49. Hahn, L., Hevesy, G., and Rebbe, O. H., Do the potassium ions inside the muscle cells and blood corpuscles exchange with those present in the plasma? Biochem. J., 1939, 33, 1549.
50. Cohn, W. E., and Cohn, E. T., Permeability of red corpuscles of the dog to sodium ion. Proc. Soc. Exper. Biol. and Med., 1939, 41, 445.
51. Rapoport, S., and Guest, G. M., Role of diphosphoglyceric acid in the electrolyte equilibrium of blood cells: studies of pyloric obstruction in dogs. J. Biol. Chem., 1939, 131, 675.
52. Rapoport, S., Stevens, C. D., Engel, G. L., Ferris, E. B., and Logan, M., The effect of voluntary overbreathing on the electrolyte equilibrium of arterial blood in man. J. Biol. Chem., 1946, 163, 411.

DIMENSIONAL, OSMOTIC, AND CHEMICAL CHANGES OF ERYTHROCYTES IN STORED BLOOD. II. EVALUATION OF SEVERAL ACID AND NEUTRAL PRESERVATION MIXTURES. EFFECT OF STORAGE AT 25° C. IN ALSEVER'S SOLUTION [1]

By S. RAPOPORT

WITH THE TECHNICAL ASSISTANCE OF MARY WING

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(Received for publication August 31, 1946)

In this communication are summarized several phases of a program aimed at assessing the value of *in vitro* methods for gauging and improving preservative techniques (1). A series of experiments is reported on the effect on the preservation of erythrocytes of variations in the extent of dilution and acidification from the original acid citrate-glucose (ACD-I) solution (2, 3). The experiments yielded only negative results. They are reported as a record for other workers in the field and because they bear on the theory of permeability of the red cell. A second phase deals with a comparison between ACD-I and Alsever's solution (4) with respect to the preservation of red cells, undertaken because of the extensive use of Alsever's solution by the United States Army in the European Theater of Operations. Because it was claimed that blood can be stored satisfactorily without refrigeration in Alsever's solution (5) an experiment on the effect of storage at 25° C. on the preservation of red cells was also included. Finally, a few data are reported on the *in vitro* changes of blood stored in De Gowin's (6) and McGill II (7) solution. These neutral solutions entail dilution of blood to the extent of 150 and 50 per cent, respectively.

Since many of the changes observed in this study are similar to those reported in the preceding communication, the report for the most part is limited to 5 indices: (*1*) hemolysis in 0.6 per cent NaCl solution, (*2*) increase of thickness, (*3*) changes in adenosine triphosphate content, (*4*) glycolysis rate and (*5*) potassium concentration of cells. In addition, calculations and data on the osmotic characteristics of the preservatives studied and the changes in corpuscular volume of erythrocytes are presented.

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Children's Hospital of Cincinnati.

METHODS AND MATERIALS

The technique of collecting and distributing the blood samples and the analytical methods used have been described previously (1).

TABLE I

Composition of acid citrate-glucose mixtures for the preservation of whole blood

Mixture	Na citrate*	Citric ac.*	Glucose*	Citr. ac. / Na citr.	pH	Vol. added to 100 ml. blood	Plasma pH
	grams per 100 ml.	*grams per 100 ml.*	*grams per 100 ml.*	*Molar ratio*		*ml.*	
ACD-I	1.33	0.47	3.00	0.49	5.03	25	7.03
ACD-IA	3.32	1.18	7.50	0.49	5.03	10	7.05
ACD-IB	1.11	0.63	3.00	0.80	4.62	25	6.82
ACD-IC	2.00	0.78	5.00	0.55	4.86	15	7.00
ACD-ID	0.88	0.80	3.00	1.27	4.17	25	6.67
ACD-IE	1.88	1.07	5.00	0.81	4.54	15	6.84
ACD-IF	1.33	0.60**	3.00	0.49		25	7.05

* Tri-sodium citrate $\cdot 2H_2O$, citric acid $\cdot H_2O$, glucose anhydrous.
** Lactic acid.

In Table I is shown the composition of the various ACD solutions studied. It may be seen that they covered a range of dilution of blood from 10 to 25 per cent, which is the most useful range and of pH from 7.0 to 6.8. In solution ACD-IF, citric acid was replaced by an equivalent amount of lactic acid with a view to exploring the possibility that accumulation of lactic acid may represent the limiting factor in the preservation of red cells. The composition of De Gowin's and McGill II solutions has been given in the literature (4, 6, 7) and is therefore omitted. A few words of comment with regard to Alsever's solution may be in order: This solution as originally described had the composition: sodium citrate 0.57,

sodium chloride 0.42, dextrose 1.87 grams per 100 ml. to be added in equal volume to blood. During the war the Baxter company modified it as follows: sodium citrate 0.80, sodium chloride 0.42, dextrose 2.05 grams per 100 ml., with the addition of 0.0075 gram of citric acid, resulting in a pH of the mixture of 6.8. This modification, designated simply as Alsever's solution in the following discussion, was the one studied in this laboratory. Later the same company modified the solution still further by adding sufficient citric acid to bring the pH to 6.0.

RESULTS

In Figure 1 is shown a comparison of McGill II and De Gowin's solution and of several variants of ACD–I solution with ACD–I mixture, portrayed graphically by plotting the values of each of the simultaneously determined indices in the solution under test against the corresponding values of the same blood in ACD–I solution. In the case of equal performance the plot will lie on a straight line equidistant from abscissa and ordinate, *i.e.*, the diagonal. Such lines are drawn in each of the graphs. For the properties of the red cell which show a tendency to increase in numerical value during storage, such as thickness, hemolysis, and potassium content of plasma, an arrow-point directed away from the origin is provided, while for those properties which tend to decrease during storage, such as the adenosine triphosphate and potassium content of cells and their glycolysis rate, the arrow is pointed toward the origin of the diagonal. The data on bloods stored in solutions inferior in preservative quality to ACD–I mixture would tend to be above the diagonal for cell characteristics which tend to increase in numerical value during storage and below it for those which tend to decrease. It may be seen that erythrocytes in McGill II solution were inferior in preservation to those in ACD–I solution with respect to hemolysis in 0.6 per cent NaCl solution, thickness, adenosine triphosphate and potassium content. With respect to glycolysis the data require additional interpretation. Erythrocytes in McGill II solution, as in other neutral mixtures, initially glycolyze at a rate greater than those in ACD–I solution, but their glycolyzing power is exhausted sooner. The values for McGill II solution lying above the diagonal line refer to blood samples after only short periods of storage, while those below the line refer to bloods stored for longer periods of time, when their glycolyzing power was exhausted, while it still persisted in a parallel blood sample in ACD–I solution. The data on the potassium content of the plasma appear to be at variance with the other indices but it should be remembered that blood stored in McGill II solution is diluted to the extent of 50 per cent, entailing an almost 2-fold dilution of the plasma. Data on hemolysis of red cells in De Gowin's solution are omitted from presentation since the osmotic behavior of erythrocytes in this solution differs markedly from that in the other solutions tested. Values for other properties of erythrocytes in this solution are also somewhat difficult to interpret because of the great increase in their cellular volume. Those charted have been corrected for the increase in volume. It appears that the cells in De Gowin's solution increased progressively in thickness during storage and that their adenosine triphosphate content and glycolyzing power became exhausted earlier than in parallel blood samples stored in ACD–I solution. The potassium content of the cells decreased at a rapid rate, probably because of the low content of electrolyte of the solution, while the potassium content of the plasma, which represents a 3.5-fold dilution of the original, was lower than in all other solutions. Solution ACD–IA, the most concentrated modification of ACD–I mixture, appears also somewhat inferior to ACD–I solution in its performance, as judged by hemolysis, thickness, and glycolysis rate. Other indices did not show significant differences. The comparatively good preservation of blood in solution IF, which contains lactic acid in amounts equal to those accumulated during extended periods of storage, although inferior to ACD–I, suggests that lactic acid *per se* is not an important deteriorative factor. None of the other solutions tested appeared significantly different in preservative quality from ACD–I solution.

In Figure 2 are shown the changes of red cells during storage at 4° and 25° C. in Alsever's solution and at 4° C. in ACD–I mixture. It may be seen that erythrocytes of blood in Alsever's solution were similar in their preservation to those in ACD solution until about the fourteenth day when both were kept at 4° C. Beyond this time they deteriorated at an increasingly rapid rate. The effects of storage of 25° C. are apparent in regard to all indices studied, storage for 2 days at this temperature being approximately equivalent to 2 weeks storage at 4° C. It may be mentioned

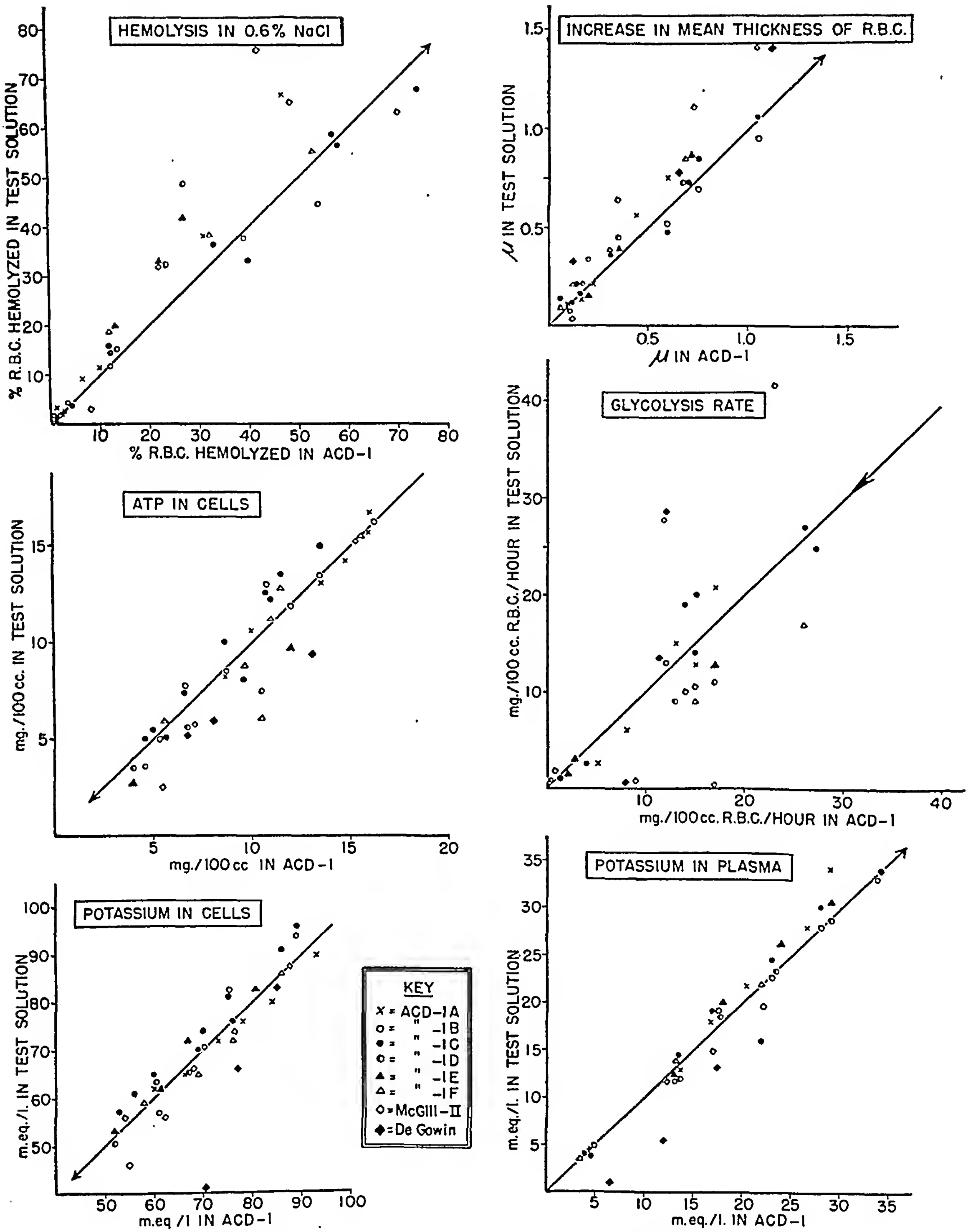

Fig. 1. Comparison of Several Acid and Neutral Citrate-Glucose Solutions with ACD-I Solution.

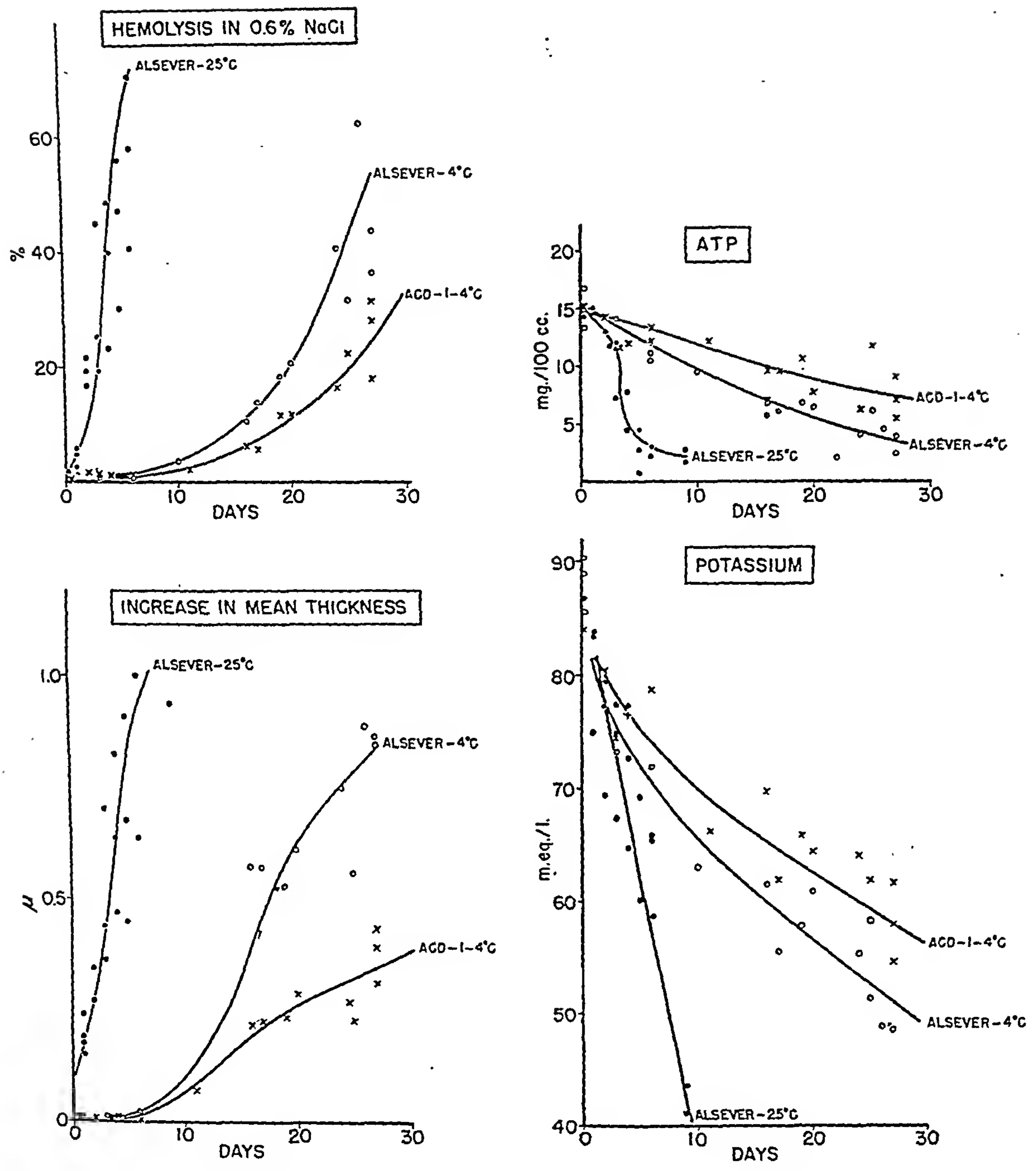

FIG. 2. CHANGES IN CELLULAR INDICES IN ALSEVER SOLUTION AT 4° AND 25° C. AND IN ACD-I SOLUTION AT 4° C.

here that even after storage periods of 10 days at room temperature spontaneous hemolysis was of small degree, indicating that this measurement is a fallacious index under such circumstances. From the data the conclusion may be drawn that non-refrigeration has a catastrophic effect on the functional state of the cell.

Osmotic characteristics of preservative solutions and corpuscular volume of erythrocytes

In Table II is given a description of the various ACD mixtures and in Table III, of the neutral preservatives from the standpoint of their osmotic and electrolyte composition. In Table IV is pre-

sented a summary of the predicted and observed values of corpuscular volume and hemoglobin concentration of erythrocytes of stored bloods in 5 acid and 3 neutral preservative solutions. As may be seen from the data, agreement between observation and theory was close for all solutions which differed in a minor degree only from the tonicity of blood. The results indicate that osmotic effects and membrane equilibria affect independently and in a strictly additive manner the corpuscular volum of erythroycytes. The constancy of the cell volume during storage in all acidified solutions and the limited increase in the neutral ones are in consonance with the hypothesis, that cations on the acid side of the iso-ionic point of hemoglobin are lost from the cell in proportion to the ionization of hemoglobin.

TABLE II

Osmolarity of various ACD preservative solutions and resulting changes in the osmotic and electrolyte concentration of blood

ACD solution	I	IA	IB	IC	ID	IE
Sodium citrate, *mM. per liter*	45	112	38	68	30	64
Sodium citrate, *m. osm. per liter*	181	450	152	272	120	255
Citric acid, *m. osm. per liter*	22	55	30	37	38	50
Glucose, *m. osm. per liter*	167	418	167	278	167	278
Total osmolarity of solution, *m. osm. per liter*	370	923	349	587	325	583
Total osmolarity of solution, *per cent of initial blood tonicity*	119	298	113	189	105	188
Effective tonicity,* *m. osm. per liter*	203	505	182	309	158	305
Effective tonicity, *per cent of initial blood tonicity*	66	163	59	100	51	98
Osmolarity of blood after mixing,** *m. osm. per liter*	324	378	319	354	314	353
Osmolarity of blood after mixing, *per cent of initial*	104	122	103	114	101	114
Electrolyte concentration of blood after mixing, *per cent of initial*	92	107	90	100	88	100

* Effective tonicity is designated the osmotic concentration of substances which permeate the cell membrane very slowly, if at all. In this instance its value was calculated on the assumption that the electrolytes, but not glucose, conform to this condition.

** Calculated on the basis of an initial concentration of 310 milliosmols per liter of water in blood, and of a water content of 0.80 gram per gram of blood.

TABLE III

Osmolarity of 3 neutral preservative solutions and resulting changes in the osmotic and electrolyte concentration of blood

Solution	Alsever	DeGowin	McGill II
Sodium citrate, *mM. per liter*	27	14.5	44
Sodium citrate, *m. osm. per liter*	108	58	174
Citric acid, *m. osm. per liter*	0.4		
Other ingredients, *m. osm. per liter*	144*		62**
Glucose, *m. osm. per liter*	114	261	120
Total osmolarity of solution, *m. osm. per liter*	366	319	356
Total osmolarity of solution, *per cent of initial blood tonicity*	118	103	115
Effective tonicity,*** *m. osm. per liter*	252	58	236
Effective tonicity, *per cent of initial blood tonicity*	81	19	76
Osmolarity of blood after mixing,† *m. osm. per liter*	341	316	328
Osmolarity of blood after mixing, *per cent of initial*	110	102	106
Electrolyte concentration of blood after mixing, *per cent of initial*	90	47	91

* Sodium chloride.

** Isotonic sodium phosphate solution pH 7.4.

*** Effective tonicity is designated the osmotic concentration of substances which permeate the cell membrane very slowly, if at all. In this instance its value was calculated on the assumption that the electrolytes, but not glucose, conform to this condition.

† Calculated on the basis of an initial concentration of 310 milliosmols per liter of water in blood, and of a water content of 0.80 gram per gram of blood.

TABLE IV

Corpuscular volume and hemoglobin concentration of erythrocytes stored in various preservative solutions

Solution	Storage period	Corpuscular volume		Corpuscular hemoglobin concentration		Plasma pH
		Observed	Predicted	Observed	Predicted	
	days	*per cent of normal**	*per cent of normal**	*per cent of normal**	*per cent of normal**	
ACD-IA	0	100	101	100	100	7.05
	20–30	100	101	99	100	6.50
ACD-IB	0	109	117	92	84	6.82
	20–30	108	117	92	84	6.41
ACD-IC	0	106	106	94	94	7.00
	20–30	107	106	94	94	6.52
ACD-ID	0	118	119	82	82	6.67
	20–30	116	119	84	82	6.32
ACD-IE	0	107	106	92	94	6.84
	20–30	107	106	92	94	6.43
Alsever	0	109	110	91	90	7.27
	20–30	114	116	88	87	6.81
McGill II	0	108	110	91	91	7.34
	20–30	115	116	87	86	6.79
De Gowin	0	142	213	74	47	7.38
	20–30	163		63		6.71

* The values in heparinized blood samples have been designated as "normal."

SUMMARY

In a study of 6 acid preservative solutions, none was found which exhibited better preservative qualities than ACD–I solution. There was some indication that one of the modifications, which was hypertonic, resulted in inferior preservation of erythrocytes. Lactic acid did not appear to be a deteriorative factor of major importance.

A comparison of Alsever's solution with ACD–I solution indicated that it possessed inferior preservative qualities. The effect of storage of blood at 25° C. in Alsever's solution was a great acceleration of deteriorative processes within the red cell.

A study of the osmotic characteristics of the various preservative solutions and of the corpuscular volume of erythrocytes indicated that addition of Alsever's and McGill II solutions resulted in only minor deviations of the electrolyte concentration of blood from the normal, while the addition of De Gowin's solution produced a decrease of the electrolyte concentration to less than ½ of normal with profound changes in the osmotic behavior of the cells. The observed corpuscular volume of erythrocytes in 5 acid and 2 neutral preservatives was in good agreement with predictions based on consideration of the osmotic and membrane equilibria of the red cell and on the assumption that red cells lose cations below the iso-ionic point of hemoglobin in amounts proportional to its ionization as a cation.

BIBLIOGRAPHY

1. Rapoport, S., Dimensional, osmotic and chemical changes of erythrocytes in stored blood. I. Blood preserved in sodium citrate, neutral, and acid citrate-glucose (ACD) mixtures. J. Clin. Invest., 1947, 26, 591.
2. Loutit, J. F., Mollison, P. L., and Young, I. M., Citric acid-sodium citrate-glucose mixtures for blood storage. Quart. J. Exper. Physiol., 1943, 32, 183.
3. Loutit, J. F., and Mollison, P. L., Advantages of a disodium-citrate-glucose mixture as a blood preservative. Brit. M. J., 1943, 2, 744.
4. Alsever, J. B., and Ainslie, R. B., A new method for the preparation of dilute blood plasma and the operation of a complete transfusion service. New York State J. Med., 1941, 41, 126.
5. Kendrick, D. B., Jr., Elliott, J., Reichel, J., Jr., and Vaubel, E. K., Supply of preserved blood to European Theater of Operations. Bull. U. S. Army M. Dept., 1945, No. 84, 66.
6. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. I. Various factors influencing hemolysis. J. A. M. A., 1940, 114, 850.
7. Denstedt, O. F., Osborne, D. E., Stansfield, H., and Rochlin, I., The survival of preserved red cells after transfusion. Anesthesiology, 1944, 5, 237.

DIMENSIONAL, OSMOTIC, AND CHEMICAL CHANGES OF ERYTHROCYTES IN STORED BLOOD. III. COMPARISON OF 3 DILUTIONS OF ACID CITRATE-GLUCOSE SOLUTION (ACD) [1]

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Previous studies have indicated that acid glucose-citrate solutions provide the best preservation available at the present time (1). The mixture proposed by Loutit and Mollison (2, 3), conventionally called ACD–I in the United States, has been most extensively studied and appears unsurpassed in its preservative qualities. It is added in amounts of 25 ml. per 100 ml. of blood. The study to be reported was undertaken with the aim of defining the minimal amount of acid citrate-glucose diluent compatible with optimal preservation of blood. With this in mind a comparison was instituted among 3 acid citrate-glucose solutions so adjusted as to produce the same degree of acidity and the same citrate concentration as is achieved with ACD mixture, even though they were added in amounts of 15, 20, and 25 ml. per 100 ml. of blood (originally designated ACD–G, H, K). On the basis of previous data which indicated that only about 0.2 gram of glucose were glycolyzed by 100 ml. of blood during the usual storage periods, the glucose content of these solutions was reduced to ½ of that in ACD–I. Since the anticipated differences between the preservatives were small compared with the variability between individual bloods, the experiment was conducted with special attention to randomization of the variables concerned. By its arrangement this study afforded an opportunity to assess the variability among different bloods treated in the same way and to compare it with the variability among several samples of one blood treated in a different way.

EXPERIMENTAL

From each of 6 healthy male volunteers samples of blood were drawn and collected aseptically in 3 different acid citrate-glucose solutions. The samples were then distributed into vials and stored in a previously described manner (1). The 18 blood samples available were tested over a period of 22 days (from the twenty-second through the forty-third day of storage), each blood sample being examined twice during the period.

The analytical methods used have been described previously, except for the procedure for estimating glycolyzing power of blood, which was modified with a view toward simplifying the measurement by determining glucose disappearance instead of lactic acid accumulation and rendering it more sensitive by increasing the pH of the blood sample during incubation.

The solutions used, the composition of which is listed in Table I, were made up on the basis of the following considerations: The concentration of glucose was adjusted so as to give a final constant increment of 0.3 gram per 100 ml. of blood mixed with the preservative solution. The concentration of sodium citrate was calculated in a similar way with the design of keeping the final concentration of citrate ion constant. In order to obtain the same pH value of about 7.0 in all bloods, the concentration of citric acid was varied so as to make the amount added to 100 ml. of blood constant. This adjustment was based on the consideration that the amount of citric acid required to bring a blood to a desired pH value is dependent on the buffering properties of blood, principally of its erythrocytes, but is unrelated to the final volume of dilution.

TABLE I

Composition of preservative solutions

Mixture	Na citrate*	Citric acid*	Glucose*	pH	Volume added to 100 ml. of blood
	grams per 100 ml.	*grams per 100 ml.*	*grams per 100 ml.*		*ml.*
ACD–15 (ACD–G)	1.98	0.78	2.30	4.90	15
ACD–20 (ACD–H)	1.58	0.59	1.80	4.96	20
ACD–25 (ACD–K)	1.33	0.47	1.50	5.03	25

* Tri-sodium citrate $\cdot 2H_2O$, citric acid $\cdot H_2O$, glucose anhydrous.

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Children's Hospital of Cincinnati.

TABLE II

Osmolarity of preservative solutions and resulting changes in the osmotic and electrolyte concentration of blood

Solution	ACD-15	ACD-20	ACD-25
Sodium citrate, *mM. per liter*	67	54	45
Sodium citrate, *m. osm. per liter*	270	215	180
Citric acid, *m. osm. per liter*	37	28	22
Glucose, *m. osm. per liter*	128	100	83
Total osmolarity of solution, *m. osm. per liter*	435	343	285
Total osmolarity of solution, *per cent of initial blood tonicity*	140	111	92
Effective tonicity,* *m. osm. per liter*	307	243	203
Effective tonicity, *per cent of initial blood tonicity*	99	78	66
Osmolarity of blood after mixing,** *m. osm. per liter*	330	317	304
Osmolarity of blood after mixing, *per cent of initial*	106	102	98
Electrolyte concentration of blood after mixing, *per cent of initial*	100	96	92

* Effective tonicity is designated the osmotic concentration of substances which permeate the cell membrane very slowly, if at all. In this instance its value was calculated on the assumption that the electrolytes, but not glucose, conform to this condition.

** Calculated on the basis of an initial concentration of 310 milliosmols per liter of water in blood, and of a water content of 0.80 gram per gram of blood.

TABLE III

Corpuscular volume and hemoglobin concentration of erythrocytes stored in 3 dilutions of ACD solution

Preservative solution	Corpuscular volume		Corpuscular hemoglobin	
	μ	*per cent of "normal"**	*grams per 100 ml.*	*per cent of "normal"**
Heparin	89.0±1.4		34.0±0.4	
ACD-15	95.3±0.4	107	31.8±0.4	94
ACD-20	97.9±0.8	109	31.3±0.4	92
ACD-25	99.5±0.8	112	30.6±0.2	90

* The values in heparinized blood samples have been designated as "normal."

The osmotic characteristics of the solutions employed are given in Table II. In Table III are summarized data on the initial corpuscular volume and hemoglobin concentration of erythrocytes in the 3 preservatives. The results are in good agreement with the hypothesis previously discussed (1).

During storage the corpuscular volumes remained unchanged at their respective values, despite increasing acidification of the blood, a finding which, if interpreted in a manner similar to that used previously, indicates escape of cations from the red cell in amounts proportional to the degree of ionization of hemoglobin as a cation.

RESULTS

From an inspection of the data in Figures 1 to 4 it is evident that the 3 dilutions of ACD solution differed in no respect from one another. Moreover there was no significant difference in any of the indices between the present series of experiments and those carried out previously with an acid citrate-glucose solution of double content of glucose (ACD-I).

The data on the regression lines of the individuals indicate that the differences between them were many-fold greater than the differences encountered between different samples of blood of 1 individual. Such data signify clearly, on the one hand, the advantages of comparing preservatives on one blood, and, on the other hand, the variability that may be expected when bloods of different individuals are compared. With *in vitro* testing the variability arises from differences among donors alone; it may be surmised that even greater differences will be found by chance alone, when *in vivo* tests are carried out under conditions where the cumulative variations of donor and recipient exert their influence.

DISCUSSION

The data presented clearly indicate that ACD-15 (ACD-G) solution is in no way inferior to the ACD solution originally described. In addition, the new solution has the advantage of entailing less dilution of blood, with the result that the final plasma protein concentration, calculated on the assumption of an initial concentration of 7 grams per 100 ml., is about 5.8 grams per 100 ml., that is, essentially the same as that in plasma of citrated blood. The explanation for the equality of the plasma protein concentrations in the 2 media lies in differences in the volume assumed by the erythrocytes. While the cells in citrate shrink by about 4 per cent, those in ACD-15 solution swell by about 7 per cent. The water shifts in opposite direction tend to cancel the existing difference of 5 per cent in the dilution of blood. A second advantage of ACD-15 solution as compared with the old acid citrate-glucose mixture is the reduced amount of glucose, which should lessen the difficulties in the preparation of dried plasma.

SUMMARY

In a comparison of 3 dilutions of ACD solution it was found that the extent of dilution of blood may be reduced to 15 ml. per 100 ml. without affecting the preservation of red cells; also, that the amount of glucose may be reduced to ½ of that in

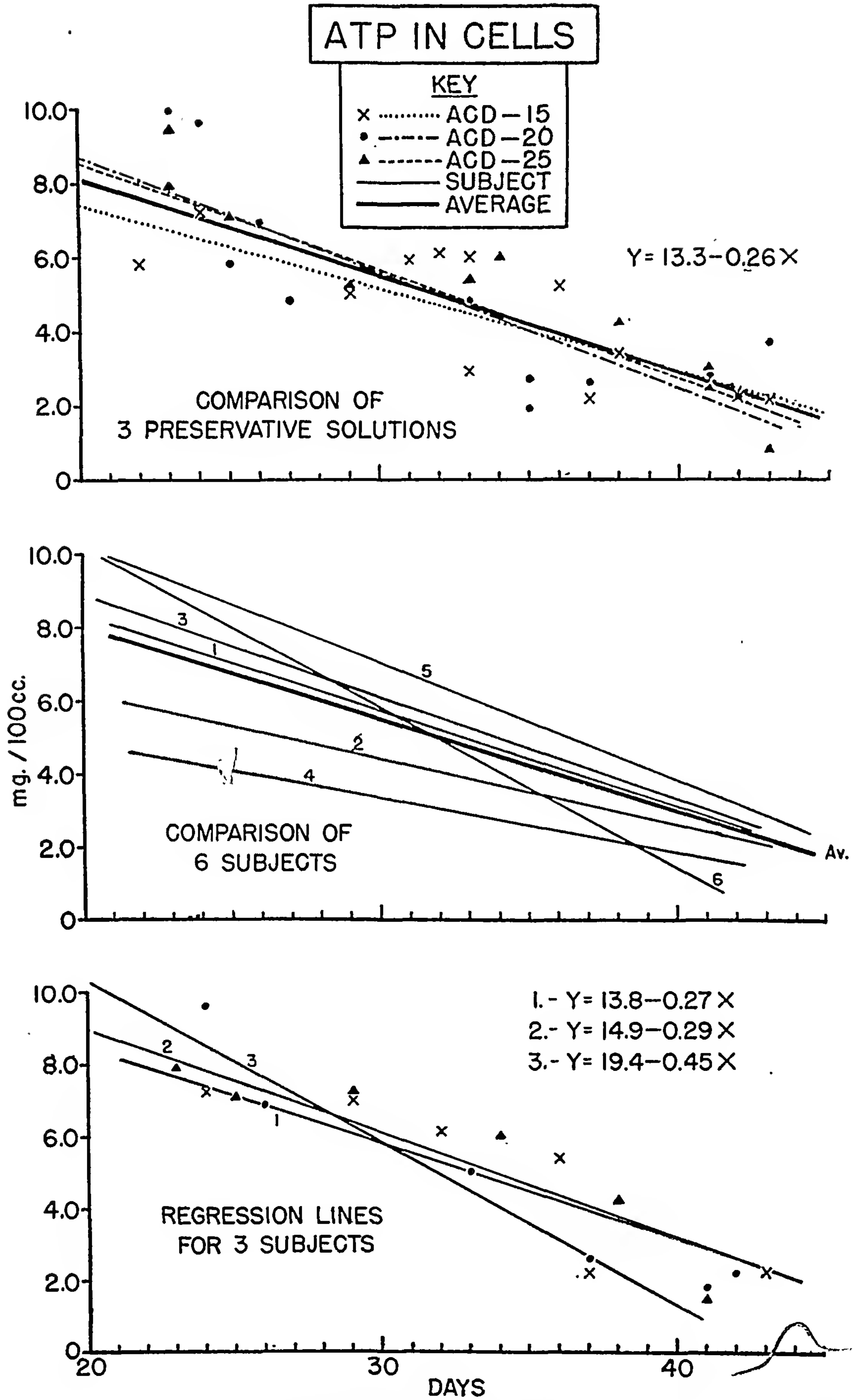

FIG. 3. A COMPARISON OF 3 DILUTIONS OF ACD–I SOLUTION WITH RESPECT TO ADENOSINE TRIPHOSPHATE CONTENT OF CELLS

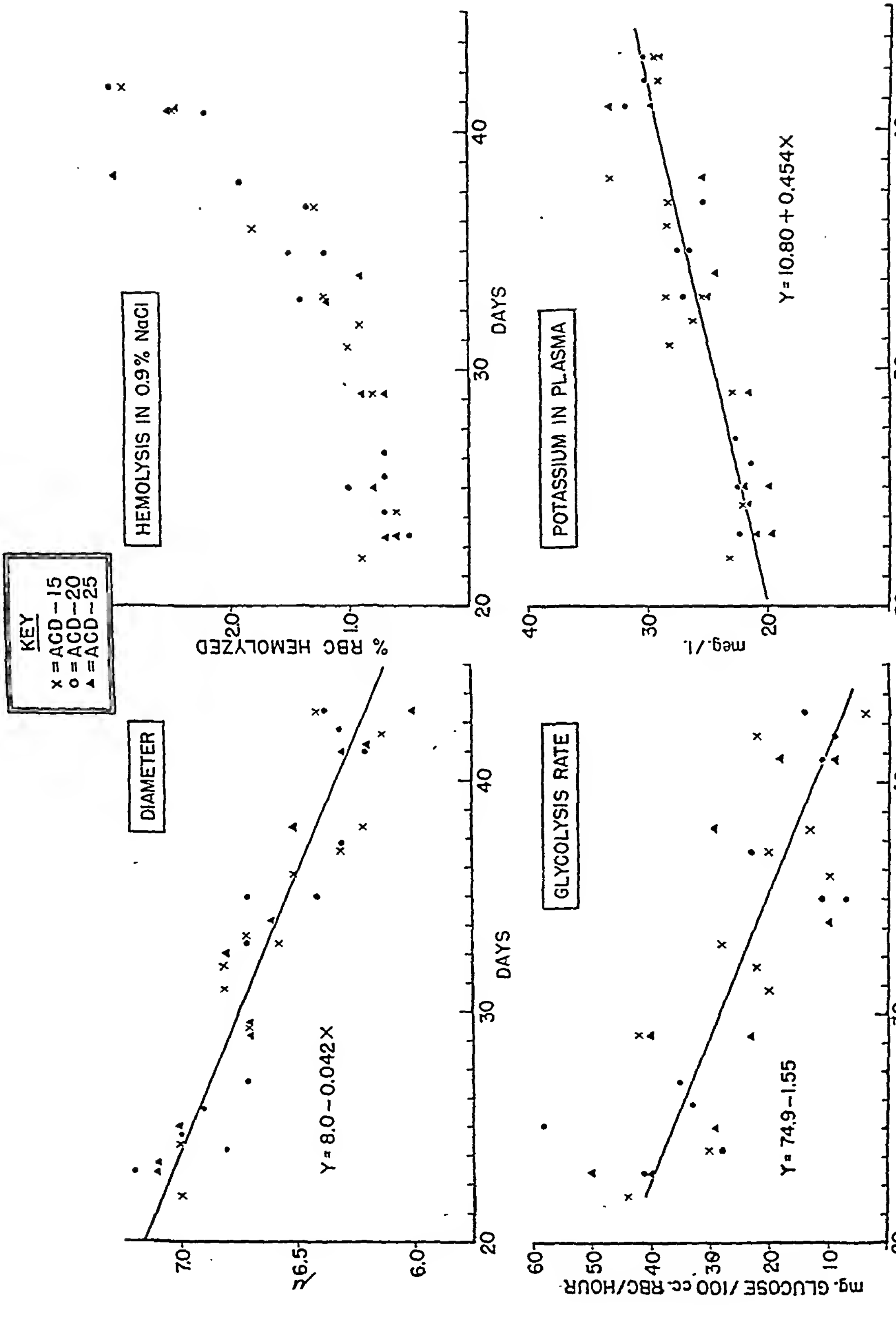

FIG. 4. CHANGES WITH TIME IN (1) DIAMETER, (2) HEMOLYSIS IN 0.9 PER CENT NACL SOLUTION, (3) GLYCOLYSIS RATE OF RED CELLS, AND (4) POTASSIUM IN PLASMA OF BLOOD IN 3 DILUTIONS OF ACD SOLUTION

the original ACD-solution. A greater variability among bloods of different individuals as compared with different blood samples of one individual has been demonstrated.

BIBLIOGRAPHY

1. Rapoport, S., Dimensional, osmotic and chemical changes of erythrocytes in stored blood. I. Blood preserved in sodium citrate, neutral, and acid citrate-glucose (ACD) mixtures. J. Clin. Invest., 1947, 26, 591.
2. Loutit, J. F., Mollison, P. L., and Young, I. M., Citric acid-sodium citrate-glucose mixtures for blood storage. Quart. J. Exper. Physiol., 1943, 32, 183.
3. Loutit, J. F., and Mollison, P. L., Advantages of a disodium-citrate-glucose mixture as a blood preservative. Brit. M. J., 1943, 2, 744.

DIMENSIONAL, OSMOTIC, AND CHEMICAL CHANGES OF ERYTHROCYTES IN STORED BLOOD. IV. CELLS SEPARATED FROM PLASMA[1]

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The possibility of utilizing erythrocytes remaining after the removal of plasma has been of increasing interest in the last years with the expanding use of plasma and its derivatives and with better understanding of the clinical use of red cells. Although several reports of the large-scale use of resuspended cells are available (1 to 9), only scant information exists on *in vitro* changes taking place in separated red cells during storage in the packed state or in resuspension. The present study, part of an investigation dealing with *in vitro* changes of stored blood, is concerned with dimensional, osmotic, and chemical changes of separated cells. In view of the fact that the American Red Cross during the war collected blood in simple citrate solution as well as in ACD solution, depending on whether the blood was to be used as such or for the preparation of plasma, both types of cells, those derived from bloods collected in simple citrate solution and those from bloods collected in ACD solution, were studied. The purposes of the study may be defined as follows: (*1*) to establish the validity of *in vitro* testing of separated red cells and (*2*) to delimit, if possible, the optimum conditions of storage and the best type of resuspension fluid for separated cells. In most instances the report will be limited to a presentation of changes of 4 indices of blood preservation: (*1*) hemolysis in 0.6 per cent NaCl solution, (*2*) changes in cellular thickness, (*3*) adenosine triphosphate content, and (*4*) glycolyzing power of erythrocytes. Complete measurements of dimensional changes, including diameter, surface and volume; of the osmotic behavior; and of the phosphorus and cation distributions were performed as described in the first paper of this series (10), but are omitted here for the sake of conciseness. The results of these studies in all instances supplemented and confirmed the data to be reported.

METHODS

Bloods were collected at the Blood Bank of the Cincinnati General Hospital in a manner earlier described (10). They were centrifuged, the plasma separated 24 hours after collection, and the blood cells promptly resuspended in an equal volume of resuspension fluid. After mixing they were distributed into a series of vials and stored at 4° C. until the time of testing. In the experiments on preservation of cells stored in the packed state, the packed cells were left undisturbed until immediately prior to testing, when they were diluted with an equal volume of 0.9 per cent NaCl solution. A series of 16 bloods were collected in the Blood Bank of the American Red Cross in Indianapolis, resuspended in the Lilly Research Laboratories, and shipped to Cincinnati by Railway Express. This series of experiments was undertaken in order to test the preservation of red cells under actual conditions of practice. Since the bloods did not differ significantly from those studied under laboratory conditions, results on them were combined with the other data. A list of the principal resuspension fluids used is given in Table I. The fluids were made up in such a way

TABLE I

Composition of mixtures for the resuspension of blood cells

Mixture	Citric acid		Total citrate	Glucose	Plasma pH
	mgm. per 100 ml. cells	*mM. per 100 ml. cells*	*mM. per 100 ml. diluent*	*grams per 100 ml. final mixture*	
C_2D_2	30	0.14	1.32	0.3	7.04
C_2E_2	60	0.29	1.32	0.3	6.96
C_2F_2	90	0.43	1.32	0.3	6.86
C_2G_2	120	0.57	1.32	0.3	6.79
C_2	0	0	1.32	0.3	
C_2E_2 Globulin 3 per cent*	60	0.29	1.32	0.3	7.04
C_2E_2 Knox gelatin 5 per cent*	60	0.29	1.32	0.3	7.02
C_2E_2 Oxypolygelatin 4 per cent*	60	0.29	1.32	0.3	7.03

The mixtures were made up with 80 ml. of 0.9 per cent NaCl and sufficient water to give a final volume of 100 ml. They were added in equal volume to separated blood cells.

* These products were brought to a pH of 7.0 by additions of NaOH or HCl before they were mixed with the other ingredients of the preservative solutions.

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Children's Hospital of Cincinnati.

as to keep the final concentration of total citrate and of dextrose constant after mixture with blood, while varying the proportions of citric acid and of trisodium citrate. The amount of citric acid was adjusted in such a manner as to cover the range between pH of 6.80 and 7.05 in the suspension after mixture with the red cells, taking into account the buffering properties of hemoglobin, the amount of lactic acid formed before resuspension, and the absence of plasma buffers. Colloids, where added, were brought to a pH of 7.0, which is close to the pH of the resuspended blood cells themselves, before they were mixed with the other ingredients of the preservative solutions. This was an important consideration, in view of the fact that the various products had varying initial pH values, 7.44, 6.74, and 5.63, for the globulin, gelatin, and oxypolygelatin preparations, respectively. Also their buffering powers varied considerably. A number of other resuspension fluids were prepared and tested but are omitted from this list since they did not differ significantly in their characteristics from the series tabulated.

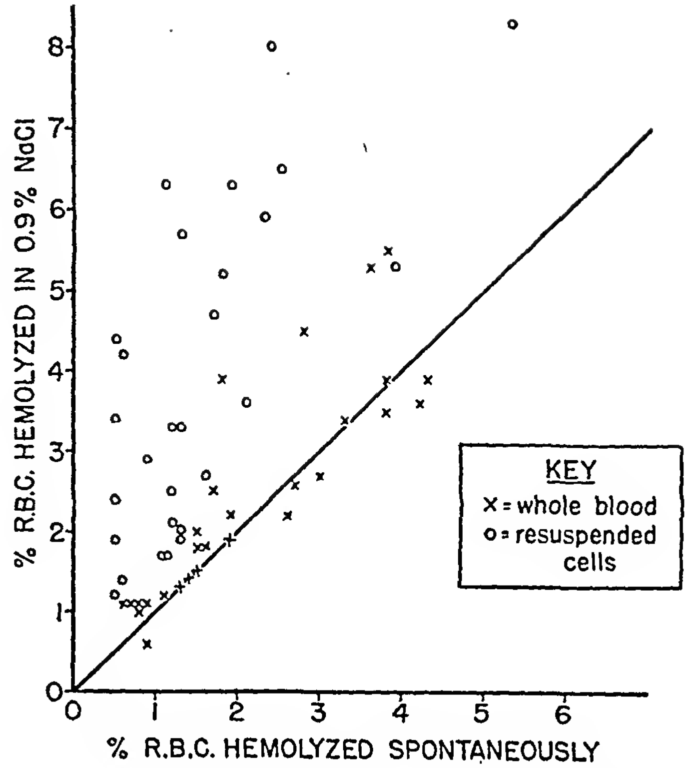

Fig. 1. Comparison of Spontaneous Hemolysis and Hemolysis in 0.9 Per Cent NaCl Solution of Resuspended Cells and of Whole Blood

RESULTS

"Spontaneous" hemolysis of resuspended cells

The measurement of so-called spontaneous hemolysis (hemolglobin in the supernatant fluid after centrifugation), used as a measure of blood preservation by some observers (11 to 14), appeared of particular interest in view of earlier indications that this test may give results in disagreement with other indices of preservation in bloods stored in solutions entailing great dilution or in suspensions of separated blood cells. In Figure 1 is presented graphically a comparison of "spontaneous" hemolysis with that in 0.9 per cent NaCl solution for erythrocytes stored as whole blood in various mixtures entailing less than 25 per cent dilution and for erythrocytes in resuspension. It may be seen that for whole blood both measurements usually gave comparable results, as indicated by the tendency of the values to group themselves around the diagonal line, while the values for all cell suspensions were above the diagonal, indicating that "spontaneous" hemolysis was much less than hemolysis in 0.9 per cent NaCl solution. It may be recalled that in earlier papers a general parallelism between hemolysis in various concentrations of NaCl, including 0.9 per cent NaCl solution and other indices of blood preservation, was demonstrated. Under the conditions described here, in resuspended cells, no correlation is found between "spontaneous" hemolysis, on the one hand, and hemolysis in 0.9 per cent NaCl solution or any of the other criteria of blood preservation, on the other. Spontaneous hemolysis under such circumstances is a fallacious index of the state of the red cell.

Optimal acidity of resuspension fluids

The beneficial effect of acidification of whole blood to a pH of about 7.0 on the preservation of erythrocytes has been established. Experiments were therefore undertaken to define the optimal amount of acid to be used in the resuspension of "citrate" cells and "ACD" cells, that is, cells derived from blood collected in simple citrate and ACD mixtures, respectively. The suspension fluids, designed to produce acidification to a varying degree and the pH values measured after suspension of the red cells, are listed in Table I. In the left-hand side of Figure 2 are presented data on the hemolysis in 0.6 per cent NaCl solution, cellular thickness, and adenosine triphosphate content of 2 suspensions of "citrate" cells after 15 days of storage. These indices are plotted against the amounts of citric acid added. It would appear from the data that 30 to 75 mgm., that is, 0.14 to 0.35 mM. of citric acid per 100 ml. of cells, provide optimal acidity for the resuspension of "ci-

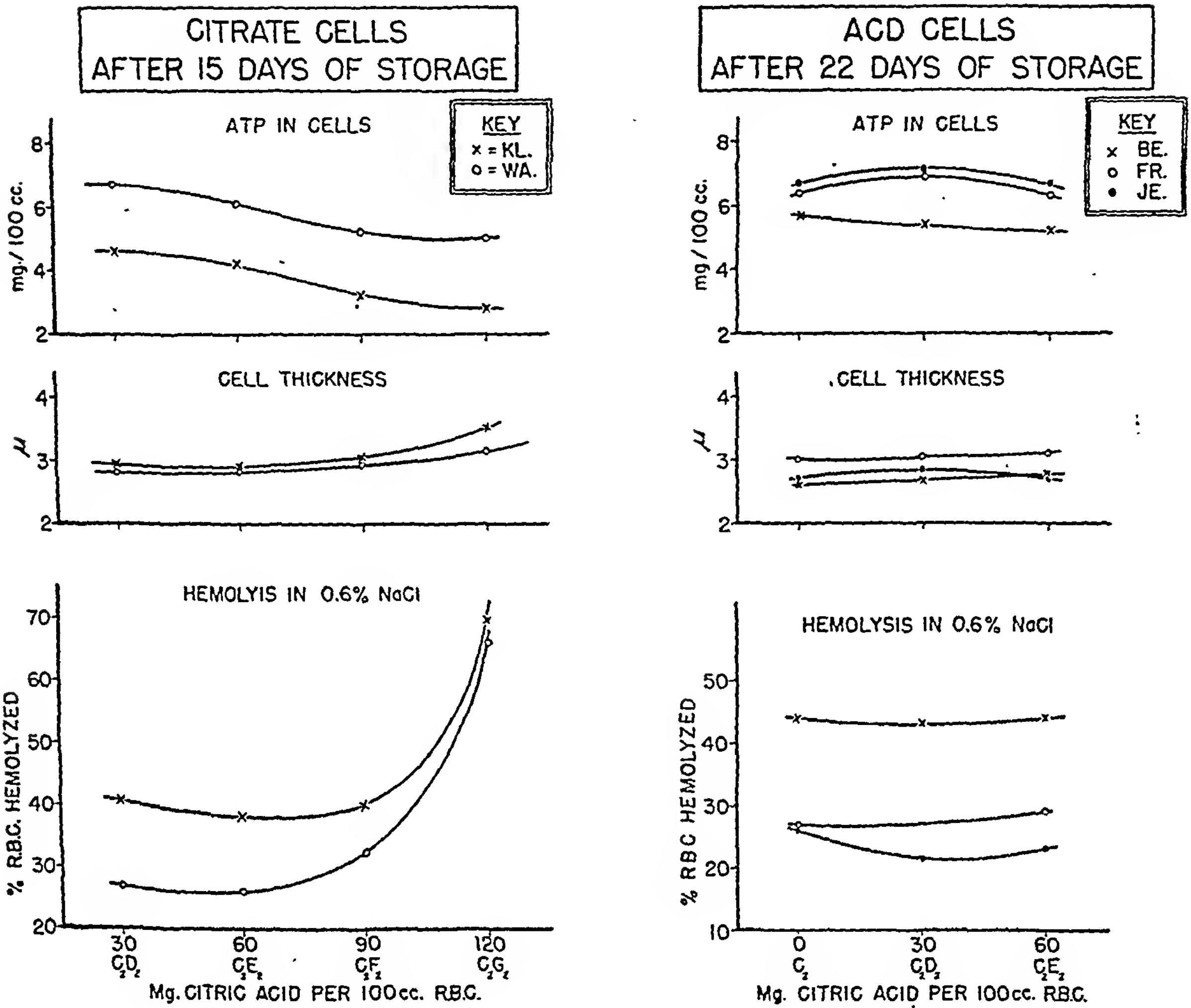

FIG. 2. CHANGES IN 3 INDICES OF "CITRATE" AND "ACD" CELLS RESUSPENDED IN SOLUTIONS OF VARYING ACIDITY

trate" cells, while greater acidification has deleterious effects.

In the right-hand side of the same figure is presented a similar experiment designed to establish the optimal amount of acid for the resuspension of "ACD" cells. It is apparent that there was no significant difference in the preservation of red cells over the entire range of acidity studied.

Comparison of cells from bloods collected in simple citrate and in ACD solution

In graphs A to C of Figure 3 are charted the individual data and in graph D the averages on the percentage of erythrocytes hemolyzed in 0.6 per cent NaCl solution after varying periods of storage and their regression lines. It may be seen that "citrate-cells," even if resuspended in ACD solution, were less well preserved than "ACD-cells." Data on changes in thickness, adenosine triphosphate content, glycolyzing power, and potassium content of erythrocytes, presented in a summary manner in Figure 4, confirm the conclusions based on the osmotic behavior of the red cells. In Table II are presented the numerical values for the regression equations of the 5 indices against time. It may be seen that the changes in all characteristics of the red cell progressed at their most rapid rate in citrate cells suspended in neutral solution and most slowly in ACD cells. It is noteworthy that the values of the various indices extrapolated to zero time approximated those actually observed in whole blood at the beginning of storage, indicating that the changes in resuspended cells tended to progress at an approximately linear rate. These find-

ings support the opinion that conditions during the first 24 hours of storage are of decisive importance for the subsequent survival of red cells. The state of preservation of resuspended cells at a given time is more variable than that of whole blood, as shown by the wide scatter of points in the graphs. There is some indication, both from inspection of the data and of the errors of the regression coefficients, that "ACD-cells" are more uniformly preserved than are "citrate-cells." Finally, it should be emphasized that all cells separated from their plasma, including ACD-cells, are less well preserved than those of corresponding whole bloods.

Comparsion of preservation of erythrocytes stored in the packed state and in resuspension

In Table III is presented a comparison in 2 samples of blood of the preservation of cells stored in the packed state and in resuspension, from which it may be seen that there was no significant difference in any of the indices between the 2 modes of storage.

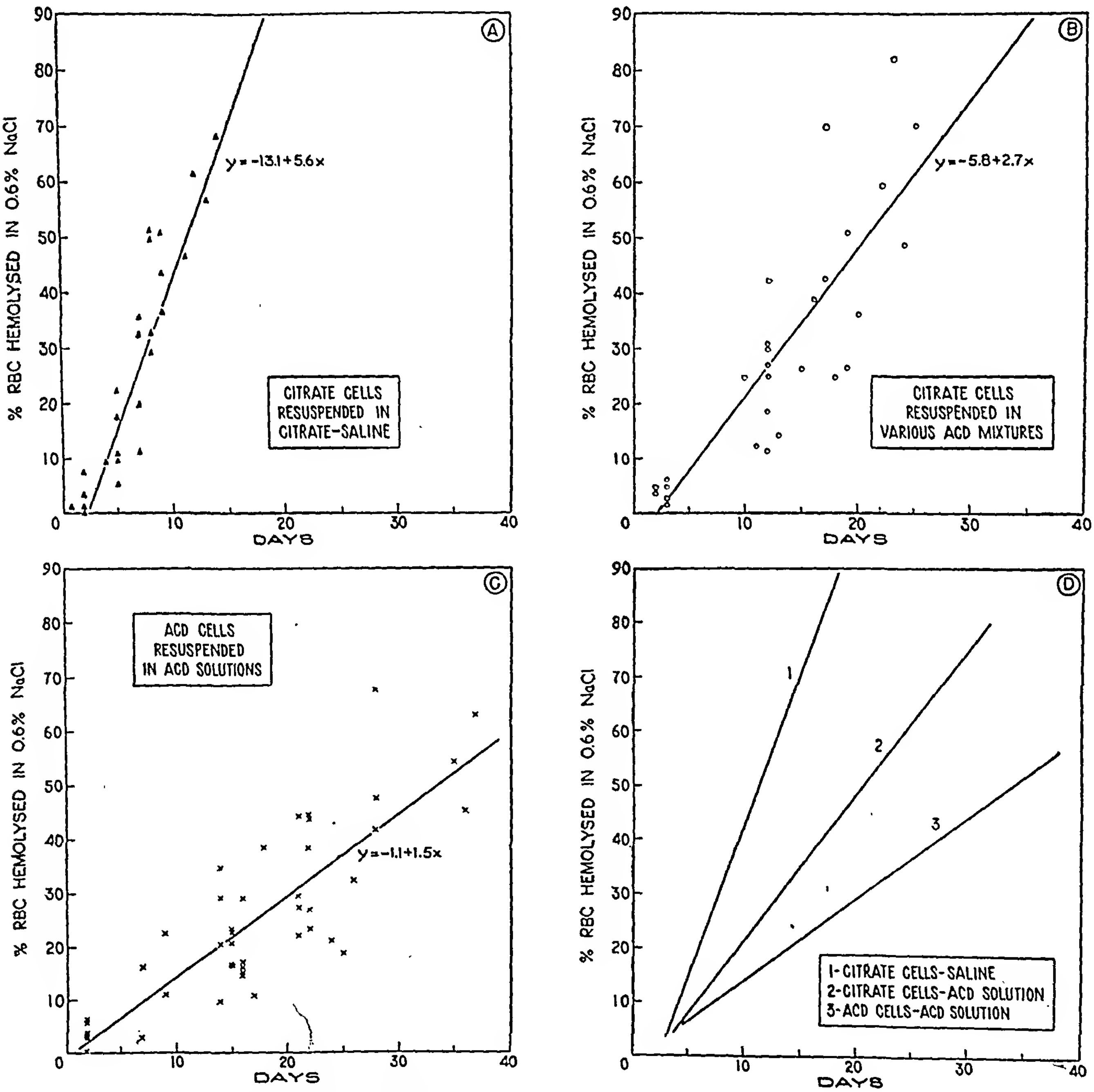

FIG. 3. COMPARISON WITH RESPECT TO HEMOLYSIS IN 0.6 PER CENT NACL SOLUTION OF CELLS DERIVED FROM BLOOD COLLECTED IN CITRATE AND ACD SOLUTION AND RESUSPENDED IN NEUTRAL AND ACID SOLUTIONS

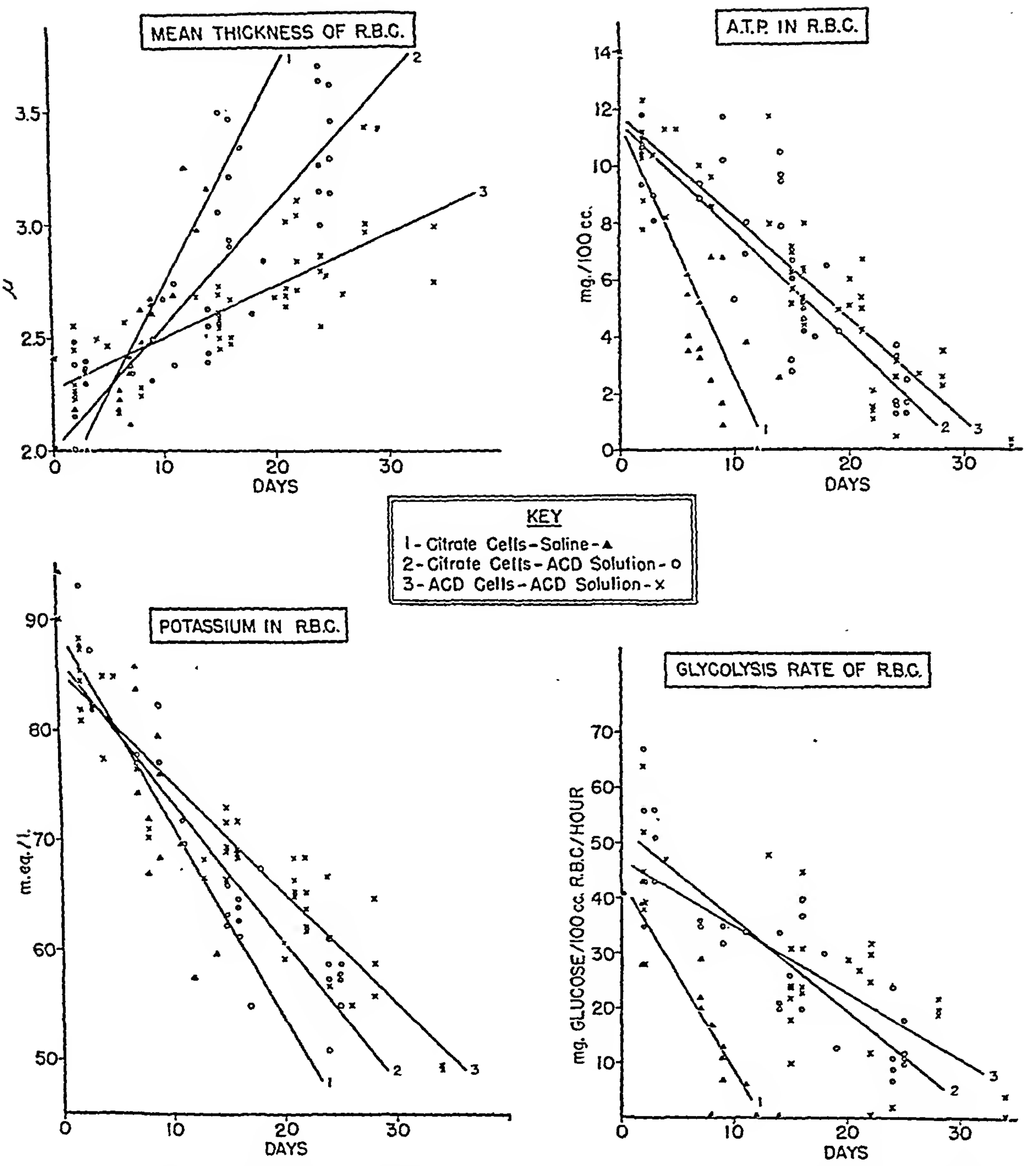

FIG. 4. COMPARISON OF "CITRATE" AND "ACD" CELLS SUSPENDED IN NEUTRAL AND ACID SOLUTION WITH RESPECT TO (1) MEAN THICKNESS, (2) ADENOSINE TRIPHOSPHATE, (3) POTASSIUM CONTENT AND (4) GLYCOLYSIS RATE

The effect of globulin, gelatin, and oxypolygelatin on the preservation of resuspended red cells

With the possibility in mind that the inferior preservation of separated red cells may be caused by the absence of the colloidal properties of the plasma proteins, the effect of several high-molecular substances on the preservation of red cells was tested. In Table IV are presented data on an experiment carried out on 2 bloods, in which the effect of globulin (Cohn fraction IV-3, 4), gelatin (Knox P16-180) and oxypolygelatin on the preservation of "citrate" cells was compared with that

TABLE II

Changes of 5 indices of preservation during storage of resuspended cells
Regression equations and their sampling errors

Cells and solution	Hemolysis of cells in 0.6 per cent NaCl	Cell thickness	ATP content of cells	Glycolysis rate of cells	Potassium in cells
	per cent	μ	*mgm. per 100 ml.*	*mgm. per 100 ml. per hour*	*m.eq. per liter*
Citrate-cells in saline	$-13.1+5.6x$ ± 0.47	$1.77+0.095x$ ± 0.009	$11.5-0.89x$ ± 0.13	$42.5-3.40x$ ± 0.43	$88.4-1.7x$ ± 0.71
Citrate-cells in ACD solution	$-5.8+2.7x$ ± 0.33	$1.99+0.054x$ ± 0.009	$11.6-0.39x$ ± 0.04	$52.7-1.66x$ ± 0.16	$86.5-1.3x$ ± 0.19
ACD-cells in ACD solution	$-1.1+1.5x$ ± 0.15	$2.27+0.023x$ ± 0.003	$11.7-0.35x$ ± 0.02	$46.8-1.20x$ ± 0.20	$85.5-1.0x$ ± 0.05

of a standard acid resuspension fluid. As may be seen from the data, no particular effect, favorable or unfavorable, was obtained by the addition of these materials. In other experiments not reported here, dextrin and small amounts of plasma protein in concentrations up to 1 per cent likewise failed to affect the preservation of cells.

The preservation of erythrocytes centrifuged and resuspended in own plasma

The data presented in Table V indicate that there was no difference in the preservation between the portion of blood that had been centrifuged and the one which had been left undisturbed, while the separated red cells showed distinctly inferior preservation. It would seem, therefore, that mechanical factors play a minor part in the determination of the preservation of the red cell.

TABLE III

Comparison of preservation of erythrocytes stored in the packed state and in resuspension

The cells studied were derived from blood collected in ACD-15 (ACD-G) solution.

Sample and condition	Storage period	Diameter	Thickness	Hemolysis in 0.6 per cent NaCl	ATP	Glycolysis rate
	days	μ	μ	*per cent*	*mgm. per 100 ml.*	*mgm. per 100 ml. per hour*
Sm. cells	20	7.2	2.35	32.3	5.1	29
Sm. suspension*	20	7.2	2.38	31.4	6.8	22
As. cells	24	7.0	2.71	27.7	3.1	30
As. suspension*	24	7.0	2.80	27.1	3.5	31

* Mixture C_2, a neutral citrate-saline-dextrose diluent, added in equal amount to the separated cells.

SUMMARY

With certain limitations *in vitro* testing is applicable to the study of resuspended red cells as well as to whole blood. A comparison of the changes of

TABLE IV

Effect of the addition of globulin, gelatin, and oxypolygelatin on the preservation of resuspended red cells

The cells studied were derived from blood collected in simple citrate solution.

Sample and mixture	Storage period	Thickness	Hemolysis in 0.6 per cent NaCl	ATP	Glycolysis rate	pH
	days	μ	*per cent*	*mgm. per 100 ml.*	*mgm. per 100 ml. of cells per hour*	
Ap. C_2E_2*	3	2.30	4.9	8.8	56	7.04
	11	2.38	12.1	8.0	34	6.66
	18	2.61	24.6	6.5	30	6.50
Ap. globulin 3 per cent (Cohn Fr. IV-3-4)	2	2.37	1.8	9.3	56	7.01
	12	2.46	10.7	8.3		6.61
	19	2.71	40.2	6 4	27	6.50
Ap. oxypolygelatin 4 per cent	11	2.48	7.1	9.2	33	6.63
	19	2.68	30.0	2.4	17	6.50
Pa. C_2E_2*	2	2.39	4.8	9.1	51	7.02
	12	2.74	26.7	6.0		6.56
	19	2.84	50.8	3.0	13	6.44
Pa. globulin 3 per cent (Cohn Fr. IV-3-4)	3	2.49	3.3	9.0	50	7.02
	11	2.55	13.0	6.9	26	6.61
	18	2.88	49.1	4.2	12	6.49
Pa. gelatin 5 per cent (Knox P16-180)	11	2.64	11.7			6.59
	19	2.98	50.8	1.9	12	6.44
Pa. oxypolygelatin 4 per cent	12	2.68	20.4			6.62
	18	2.96	47.9	3.0	11	6.49

* The composition of this mixture is given in Table I.

TABLE V

The preservation of erythrocytes, centrifuged and resuspended in own plasma

The blood studied was collected in ACD solution. One portion was left undisturbed, another one was centrifuged for 2 hours and resuspended, and a third one was centrifuged, the plasma removed and replaced by an acid crystalloid resuspension solution. Measurements of preservation were carried out after 17 days of storage.

Condition of sample	Diameter	Thickness	Hemolysis in 0.9 per cent NaCl	Hemolysis in 0.6 per cent NaCl	ATP	Potassium	pH
	μ	μ	*per cent*	*per cent*	*mgm. per 100 ml. of cells*	*m.eq. per liter of cells*	
Undisturbed	7.2	2.60	1.1	33.2	6.2	71.5	6.58
Centrifuged and resuspended in own plasma	7.2	2.65	1.0	35.7	6.8	70.4	6.61
Resuspended in crystalloid solution	6.8	3.11	2.3	49.1	4.4	63.2	6.55

the various indices *in vitro* indicates that they maintain similar relationships with each other as they do in whole blood. Even under the best conditions the preservation of separated red cells is inferior and much more variable than that of the cells of corresponding whole blood. Cells derived from bloods collected in ACD solution are better preserved than those derived from bloods collected in citrate solution. The preservation of cells from citrate blood may be improved by resuspension in acidified solutions, but even then they are not so well preserved as ACD cells. Such data indicate that processes taking place in the first 24 hours of storage before the cells are separated have a determining influence on the course of the subsequent preservation. The optimal acidity for the preservation of citrated cells may be defined within the limits of 0.14 to 0.35 mM. of citric acid per 100 ml. of cells. ACD cells may be conveniently and advantageously stored in packed form. The cause of the inferior preservation of separated red cells is unknown at present. Addition of globulin or gelatin products does not appear to improve their preservation.

I should like to thank Dr. C. G. Culbertson and Mrs. H. Zerfas of the Blood Donor Service of the American Red Cross in Indianapolis and Mr. W. A. Jamieson of the Lilly Research Laboratories for their cooperation in obtaining samples of blood cells.

BIBLIOGRAPHY

1. MacQuaide, D. H. G., and Mollison, P. L., Treatment of anemia by transfusion of concentrated suspensions of red cells. Brit. M. J., 1940, 2, 555.
2. Williams, G. E. O., and Davie, T. B., Preparation and use of concentrated red cell suspensions in treatment of anemia. Brit. M. J., 1941, 2, 641.
3. Watson, L., Red-cell suspension transfusions. Lancet, 1943, 1, 107.
4. Evans, R. S., The use of concentrated red cells as a substitute for whole blood in transfusion therapy of anemia. J. A. M. A., 1943, 122, 793.
5. Taylor, E. S., Thalhimer, W., and Cooksey, W. B., The organization of a red blood cell transfusion service. J. A. M. A., 1944, 124, 958.
6. Binder, M. L., and Klein, A., Concentrated red cell transfusions. Am. J. M. Sc., 1944, 208, 95.
7. Cooksey, W. B., and Horwitz, W. H., Use of salvaged red cells. J. A. M. A., 1944, 124, 961.
8. Thalhimer, W., and Taylor, E. S., The transfusion of centrifuged human type O cells resuspended and stored in 10 per cent corn syrup. J. A. M. A., 1945, 127, 1096.
9. Bagdasarov, A., Blood transfusion in U.S.S.R. Brit. M. J., 1942, 2, 445.
10. Rapoport, S., Dimensional, osmotic and chemical changes of erythrocytes in stored blood. I. Blood preserved in sodium citrate, neutral, and acid citrate-glucose (ACD) mixtures. J. Clin. Invest., 1947, 26, 591.
11. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. I. Various factors influencing hemolysis. J. A. M. A., 1940, 114, 850.
12. Muether, R. O., and Andrews, K. R., Studies on stored blood. II. Effect of storage on human blood. Am. J. Clin. Path., 1941, 11, 314.
13. Alsever, J. B., and Ainslie, R. B., A new method for the preparation of dilute blood plasma and the operation of a complete transfusion service. New York State J. Med., 1941, 41, 126.
14. Kendrick, D. B., Jr., Elliott, J., Reichel, J., Jr., and Vaubel, E. K., Supply of preserved blood to European theater of operations. Bull. U. S. Army M. Dept., 1945, No. 84, 66.

THE OSMOTIC RESISTANCE (FRAGILITY) OF HUMAN RED CELLS[1]

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The degree of resistance of red cells to a decrease of the salt content of their environment has long been used experimentally as one measure of their viability and clinically as a diagnostic characteristic. The experimental basis for this measure has been the fact that when any red cell, of a population of red cells, reaches a certain volume (the hemolytic volume, Jacobs [1]), the hemoglobin of that cell diffuses to equilibrium inside and outside the cell, usually without rupture of the plasma membrane of the cell. Since any given population of red cells possesses individual cells which differ from one another in the volume at which hemoglobin is permitted to diffuse out, the osmotic resistance of such a population of red cells is assumed to follow a normal probability distribution. In fact, the "Fragility Test"[2] is based on this assumption. This is a reasonable assumption for freshly collected red cells of normal man (Figure 1) (Hunter [2], Guest and Wing [3]).

Clinical data on fragility and frequently laboratory experimental data have been collected by visual selection of the salt concentration at which the maximal and minimal degree of hemolysis occurs. Unfortunately this method of observation means that the data have been obtained on the least accurate parts of the osmotic resistance curve, namely at its extremes. In these extreme regions of the curve, small differences in the degree of hemolysis correspond with large differences of salt concentration (Figure 1); and, hence, one would expect divergent results, particularly since subjective, visual observation of the degree of hemolysis has been used.

During the course of studies on the storage of whole blood it became necessary to determine accurately the variation in osmotic resistance of stored red cells. A method was devised for this purpose which in some respects is similar to that of Hunter (2). It took into account, however, several factors which markedly affect the normal osmotic resistance (Jacobs and Parpart [4]). The chief factor is the pH of the sodium chloride solutions (Figure 1). It is obvious from these curves that it is necessary to buffer the salt solutions. For this reason a stock solution was prepared containing the following: 180.00 grams NaCl, 27.31 grams Na_2HPO_4, 3.74 grams NaH_2PO_4, all made up to 2 liters with distilled water. This "Stock NaCl–PO_4" solution is equivalent to a 10 per cent solution of NaCl and will keep for a number of months.

The salt concentrations for measurement of the osmotic resistance were prepared from this stock solution as follows:

A calibrated 50 ml. burette was filled with the "Stock NaCl–PO_4" solution and the volumes given in column 2, Table I, run into a 250-ml. volumetric flask. This was made up to the 250-ml. mark with distilled water and put into a glass stoppered pyrex bottle labeled with the salt concentration shown in column 1. The pH of these salt

TABLE I

Column 1 NaCl–PO_4	Column 2 Stock NaCl–PO_4 made up to 250 ml.	Column 3 Stock NaCl–PO_4 made up to 250 ml. "Complementary Solutions"
per cent	*ml.*	*ml.*
0.8	20.00	30.00
0.75	18.75	31.25
0.70	17.50	32.50
0.65	16.25	33.75
0.60	15.00	35.00
0.55	13.75	36.25
0.50	12.50	37.50
0.45	11.25	38.75
0.40	10.00	40.00
0.35	8.75	41.25
0.30	7.50	42.50
0.25	6.25	43.75
0.20	5.00	45.00
0.10	2.50	47.50

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Princeton University.

[2] The term "Osmotic Resistance" will be used throughout this paper in a sense that is somewhat analagous to the term "Fragility." It is used because it more adequately defines the physical changes occurring in red cells under these conditions than does the term fragility.

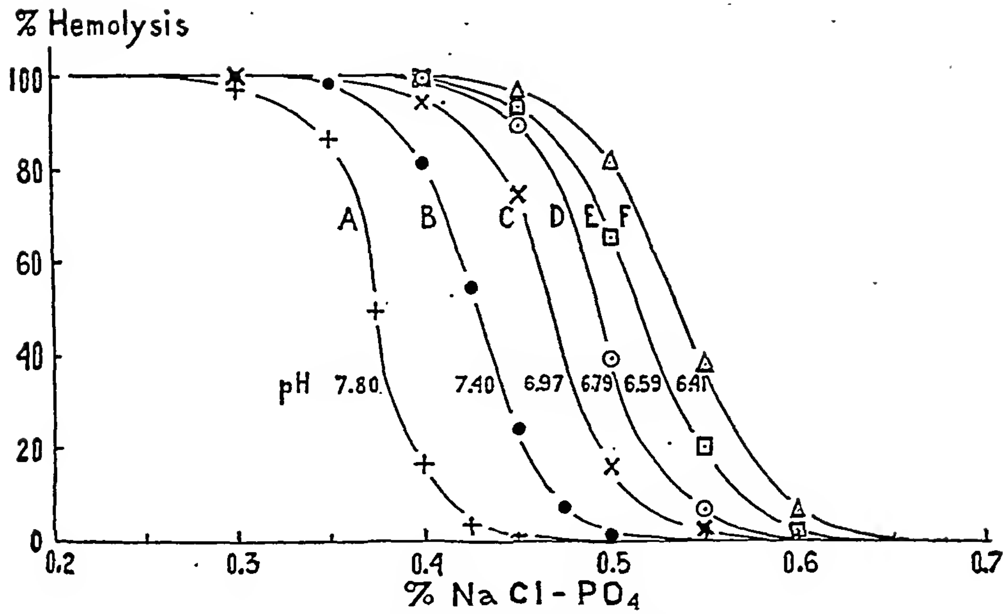

FIG. 1. OSMOTIC RESISTANCE CURVES OF NORMAL HUMAN BLOOD (MALE, WHITE) AS AFFECTED BY THE pH OF THE SALT SOLUTIONS

The per cent $NaCl-PO_4$ is equivalent to per cent NaCl of the usual fragility plot. Curve B is the average of determinations on the blood of 12 normal young men, no individual sample of which varied by more than 0.015 per cent NaCl from the average.

solutions is 7.40. The remainder (as shown in column 3, Table I) of each 50 ml. of Stock $NaCl-PO_4$ used for a particular salt concentration was also run into a 250-ml. flask made up with distilled water and stored in pyrex bottles labeled $C_{0.8}$; $C_{0.75}$, etc.

The osmotic resistance series was set up by transferring 5 ml. of each of the salt concentrations to centrifuge tubes and adding 0.020 ml. of citrated blood (4 mgm. $Na_3C_6H_5O_7 \cdot 2\,H_2O$, added dry, per ml. blood). The transfers were made with a 5-ml. syringe fitted with a 3-inch No. 18 needle, and the blood was added from a hemoglobin pipette. The blood and the salt solution were equilibrated for 45 minutes. At this time the hemolytic process was stopped by the addition of the corresponding "Complementary Solution" ($C_{0.8}$; $C_{0.75}$, etc.), column 3, Table I. Thus, the osmotic pressure around the unhemolyzed cells was returned to normal tonicity and further hemolysis stopped.

The percentage hemolysis was then determined either by a calibrated photoelectric densitometric method (Parpart [5]); or by centrifuging of the blood-salt solutions for 5 minutes at 1,500 r.p.m.; removing[3] the supernatant cell-free hemoglobin solution and determining its concentration in a photoelectric colorimeter at the wave length 540 mμ.

Calibration for the per cent hemolysis was run on the same sample of blood. Where a photoelectric colorimeter is used to read the amount of hemoglobin in the supernatant solution, the calibration is performed as follows:

Transfer 5 ml. of 0.1 per cent $NaCl-PO_4$ solution to a test tube and add 0.020 ml. of blood; allow to stand for 15 minutes, then add 5 ml. of $C_{0.1}$. Mix well and read in colorimeter. This reading gives the value for 100 per cent hemolysis. If the hemoglobin solutions in the particular photoelectric system used have been shown to obey Beer's Law, the per cent hemolysis in the various salt solutions may be calculated as follows:

$$\frac{D_{\text{exp.}}}{D_{100\text{ per cent}}} \times 100 = \text{per cent hemolysis}$$

where the optical density, D, equals the $\log_{10}$ of the reciprocal of the transmission value obtained in the colorimeter. $D_{100\text{ per cent}}$ is that for the calibration solution; $D_{\text{exp.}}$ is that for the supernatant fluid of any particular blood-salt solution.

In the practical use of this method one soon learns that not all of the 14 salt solutions recommended in Table I need be used for every sample of blood. Occasionally, finer gradations of salt concentration (*e.g.*, 0.325, 0.375, 0.425, 0.475 etc.), may be needed. Such intermediate salt concentrations are readily prepared by mixing equal volumes of the salt solution in Table I above and below the desired concentration.

Two very useful criteria of osmotic resistance come out of data obtained in this way. By using

[3] A syringe fitted with a long No. 18 needle, whose end is bent up to form a J, is convenient for removing the hemoglobin solution.

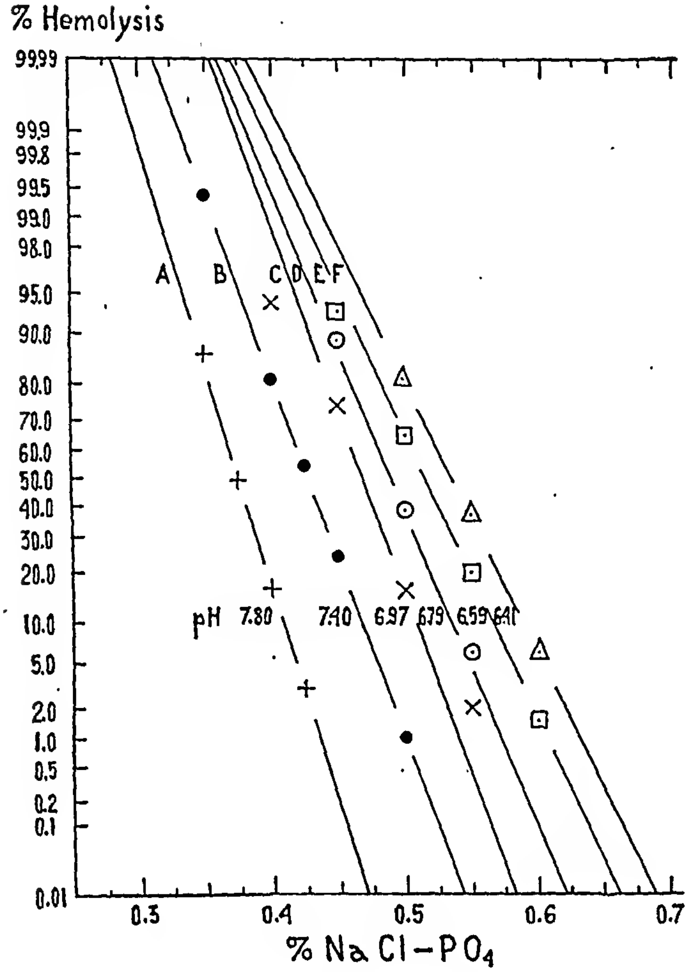

FIG. 2. A PROBABILITY PLOT OF THE OSMOTIC RESISTANCE CURVES OF FIGURE 1

This figure illustrates the ease with which the spread and the median of osmotic resistance may be determined graphically.

Probability Paper,[4] (Hunter [2]), the spread of the osmotic resistance and the median may be obtained graphically (Figure 2). It shoud be emphasized that accurate data on the spread and the median can be obtained only if 3 or more of the salt concentrations used give degrees of hemolysis between 10 per cent and 90 per cent (*i.e.,* in the critical concentration range).

The importance of stopping the hemolytic process at a fixed time by the addition of the complementary solution, is illustrated in Figure 3. These data were obtained by putting 0.020 ml. of blood into 5-ml. samples of salt solution which had been brought to and were kept at the temperatures given and at the time indicated on the abscissa, adding 5 ml. of the complementary solution, and determining the per cent hemolysis in the manner described. Red cells equilibrated for 45 minutes at 20° ± 0.1° C. in a hypotonic salt solution causing about 20 per cent hemolysis, are very near the equilibrium per cent of hemolysis. At higher temperatures the equilibrium is attained more rapidly (5 to 10 minutes at 40°); the Q_{10} is of the order of 2.6. At temperatures below 20° C. a time increasingly longer than 45 minutes must be allowed for attainment of equilibrium, particularly for blood in salt solutions which cause less than 50 per cent hemolysis. At 10° C. equilibrium is reached after about 2 hours. When a salt solution of low concentration, which brings about complete hemolysis, is used the equilibrium is reached in a few minutes even at 10° C., (Jacobs, Glassman and Parpart [6]).

If room temperatures fluctuate widely, consistent data on the osmotic resistance can be obtained only by equilibrating the blood-salt solutions in a water bath the temperature of which does not vary by more than a degree. A further, though less striking, illustration of this point is presented in the data on the spread and median of the osmotic resistance of normal human blood at 10°, 20°, 30° and 40°, as given in a probability plot (Figure 4). As would be expected the osmotic resistance increases (fragility decreases), with increasing temperature (Jacobs and Parpart [4]). Thus, the spread at 10° is from 0.330 to 0.531 per cent NaCl–

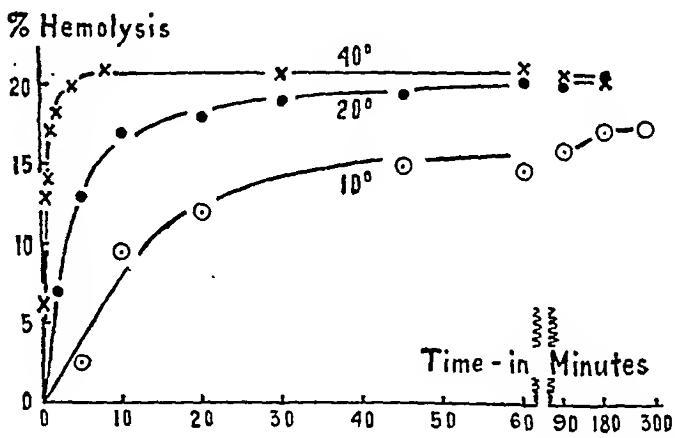

FIG. 3. ILLUSTRATES THE RATE AT WHICH THE EQUILIBRIUM DEGREE OF HEMOLYSIS IS ATTAINED AT 3 TEMPERATURES

Salt concentrations were chosen (from data in Figure 4) so that the equilibrium degree of hemolysis (17 to 20 per cent) would be approximately the same at the 3 temperatures. Details are given in the text.

[4] Keuffel and Esser Probability Paper No. 359–23.

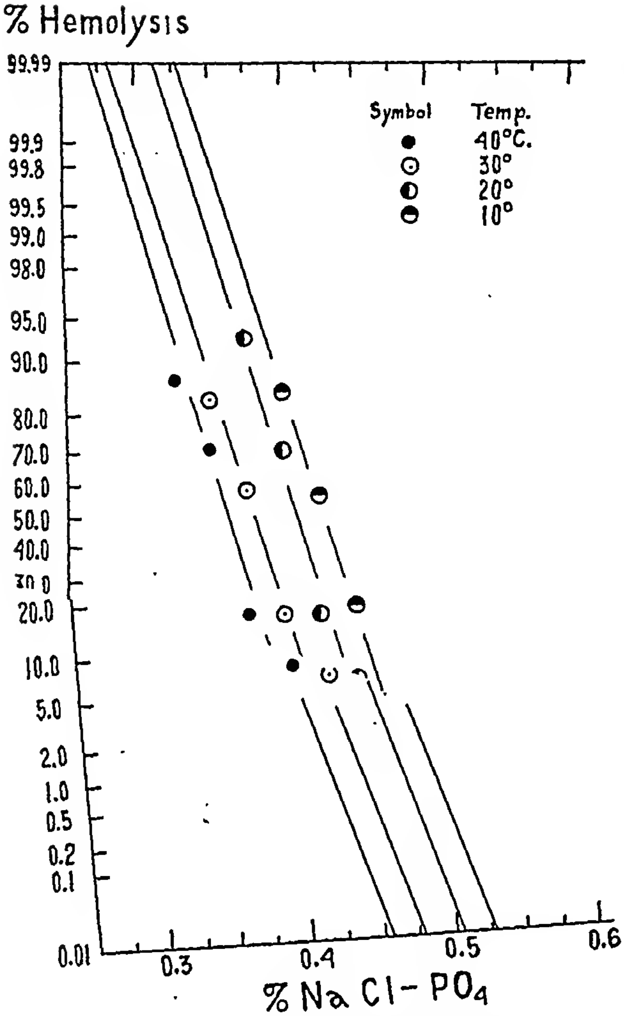

FIG. 4. ILLUSTRATES THE EFFECT OF TEMPERATURE ON THE SPREAD AND MEDIAN OF THE OSMOTIC RESISTANCE OF NORMAL HUMAN BLOOD

PO_4, while that at 40° is from 0.270 to 0.455; and the medians are 0.430 and 0.361 respectively.

It is obvious that temperature markedly affects the time at which the equilibrium degree of hemolysis may be attained, especially for blood-salt solutions in which low degrees (20 per cent) of hemolysis occur (Figure 3). Equally pertinent is the fact that once the equilibrium has been reached the temperature at which it is attained is a factor in determining the amount of hemolysis that will take place in a salt solution in the critical concentration range for solutions causing between 10 and 90 per cent hemolysis (see Figure 4).

The data on the effect of pH on the osmotic resistance (Figures 1 and 2) clearly demonstrate that this factor must be adequately controlled if reliable results are to be obtained. Examination of the data in Figure 2 will show that a change of pH by 0.1 of a pH unit is equivalent to altering the salt concentration by 0.01 per cent. The extreme variation for 12 normal adult males (curve B, Figure 2), is 0.02 per cent of salt concentration; if the pH had not been carefully controlled, this variation could have been caused by a shift of only 0.2 of a pH unit.

The osmotic resistance of blood may be greatly altered if it is collected under conditions where a large volume of an isosmotic glucose solution is added to it or where the salt content of the fluid surrounding the cells is reduced. Thus the red cells of blood collected by the method of De Gowin (7) have their median osmotic resistance (M.O.R.) at 0.800 per cent NaCl within 3 hours after collection, instead of at the normal M.O.R. of 0.425 per cent NaCl (curve B, Figure 1). On of pure ... rapid ion exchanges and resuspended in solutions Parpart [8]) which lead to an increase ... dergo osmotic resistance. In short, the environment ... the red cells prior to measuring their resistance to changes in osmotic pressure must be considered in the analysis of their behavior.

SUMMARY

1. The factors which affect the measurement of the osmotic resistance of red cells (human) are analyzed. A method for such measurements is presented.

2. The factors: pH, temperature, rate of attainment of equilibrium, and chemical environmental changes each markedly influence the measurement of osmotic resistance. The magnitude and direction of their effects are considered.

BIBLIOGRAPHY

1. Jacobs, M. H., The complex nature of the effects of temperature on the rates of certain biological processes. The Am. Naturalist, 1928, 62, 289.
2. Hunter, F. T., A photoelectric method for the quantitative determination of erythrocyte fragility. Clin. Invest., 1940, 19, 691.

3. Guest, G. M., and Wing, M., Osmometric behavior of normal human erythrocytes. J. Clin. Invest., 1942, 21, 257.
4. Jacobs, M. H., and Parpart, A. K., Osmotic properties of the erythrocyte. II. The influence of pH, temperature, and oxygen tension on hemolysis by hypotonic solutions. Biol. Bull., 1931, 60, 95.
5. Parpart, A. K., The permeability of the mammalian erythrocyte to deuterium oxide. J. Cell. & Comp. Physiol., 1935, 7, 153.
6. Jacobs, M. H., Glassman, H. N., and Parpart, A. K., Osmotic properties of the erythrocyte. VII. The temperature coefficients of certain hemolytic processes. J. Cell. & Comp. Physiol., 1935, 7, 197.
7. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. I. Various factors influencing hemolysis. J. A. M. A., 1940, 114, 850.
8. Jacobs, M. H., and Parpart, A. K., Osmotic properties of the erythrocyte. VI. The influence of the escape of salts on hemolysis by hypotonic solutions. Biol. Bull., 1933, 65, 512.

WHOLE BLOOD PRESERVATION; A PROBLEM IN GENERAL PHYSIOLOGY.[1] AN *IN VITRO* ANALYSIS OF THE PROBLEM OF BLOOD STORAGE

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A study of the storage of whole blood presents numerous problems in the field of cellular physiology. An understanding and solution of these problems can be of great aid in prolonging the viability of red cells of stored blood.

A major part of the problem centers around experimental studies on the influence of a number of environmental factors on survival. The more important factors are: temperature, range and optimum; degree of dilution; tonicity of the diluent; composition of the diluent; ion balance, maintenance of the colloidal osmotic pressure; pH range and optimum; buffer systems; glucose and rate of glycolysis; acid metabolite formation.

There have been many studies of the effect of some of these environmental factors on blood storage. In most cases, however, they have been studied as isolated, incomplete events by methods that frequently did not allow proper judgment of the state of preservation of the red cells. More recently, several excellent studies along these lines have appeared (Loutit [1]).

It is essential that an *in vitro* study of the blood preservation be accomplished by a thorough examination of a number of carefully controlled experimental criteria. We have found that the correlation obtained from the following *in vitro* methods permits one to arrive at a reasonable judgment of the state of preservation of the stored red cells and to predict the *in vivo* survival of such cells. Such predictions have been confirmed by *in vivo* tests (Gibson, *et al* [2]).

IN VITRO METHODS EMPLOYED IN THESE STUDIES

1. *The degree of spontaneous hemolysis,* obtained spectrophotometrically on the plasma after thorough resuspension of the cells and subsequent separation in an air turbine (Chase, *et al* [3]).

2. *The mean and spread of the osmotic resistance,* determined by an experimental analysis of the full curve of osmotic resistance, in buffered salt solution at fixed pH and temperature (Parpart, *et al* [4]).

3. *Rate of loss of potassium from the red cells,* obtained by potassium analysis on red cells and plasma (Weichselbaum, Somogyi, and Rusk [5]).

4. *The rate of glycolysis,* as determined by the rate of disappearance of glucose from the stored blood (Hoffman [6]).

5. *Rate and amount of lactic acid formation* (Barker and Summerson [7]).

6. *Changes in hydrogen ion concentration of the plasma,* by glass electrode measurements.

7. *Hematocrit and cell volume changes,* determined by an accurate (standard deviation of 0.86 per cent) air turbine method (Parpart and Ballentine [8]).

8. *Initial total hemoglobin, methemoglobin formation and cell counts,* methods of Chase, *et al* (3) and Parpart (9).

9. *Rate of exchange of anions across the membrane of the red cells,* measured photoelectrically (Parpart [10]).

10. *Rate of passage of non-toxic organic molecules into the red cells,* measured photoelectrically (Parpart [10]).

All of the data reported in this paper were obtained on blood samples collected by venipuncture from healthy human males (18 to 40 years of age).

RESULTS

Appropriate correlations between these criteria have served as a satisfactory index of the state of preservation obtained in the various procedures we have applied to stored blood. The results of these studies will be outlined under separate headings, though it is important to realize that this separation is purely for convenience. Only full cognizance of the interaction of the various variables studied will allow for proper comprehension of the factors involved in blood storage.

1. Optimal temperature for preservation.

Studies have been made on whole blood samples stored at different temperatures under a variety of

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Princeton University.

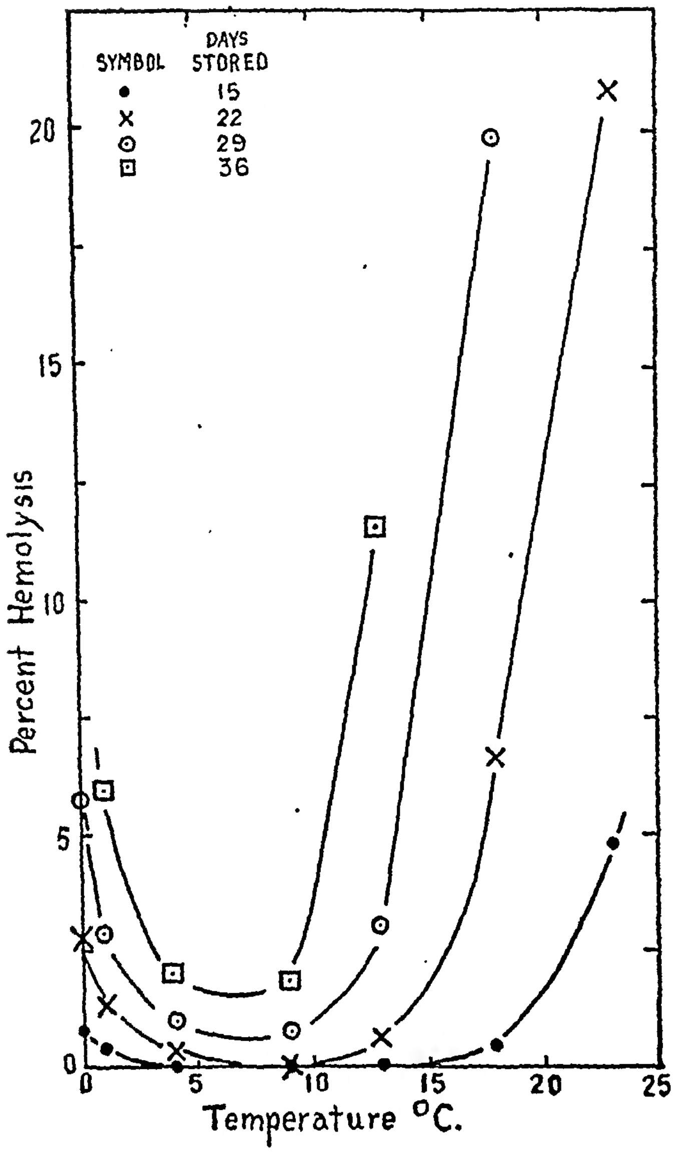

FIG. 1. EFFECT OF TEMPERATURE ON THE SPONTANEOUS HEMOLYSIS OF STORED BLOOD

experimental conditions (*e.g.* diluted, defibrinated, citrated, normal, and added glucose).

Figures 1, 2, and 3 illustrate the typical influence of temperature on the survival of stored human blood. These data were obtained on 270 ml. of blood collected in a bottle containing 30 ml. of 3.25 per cent $Na_3C_6H_5O_7 \cdot 2\,H_2O$ (0.11 M). This blood was then diluted to 70 per cent of normal whole blood by the addition of an isosmotic solution (5.4 per cent) of glucose and within 30 minutes of collection stored at the temperature indicated in the figures.

Figure 1 gives the rate of spontaneous hemolysis with respect to temperature and length of storage; Figure 2 presents the data on the mean osmotic resistance and Figure 3 illustrates the effect on the ability of the red cells to retain their normal potassium content. Four complete repetitions of these experiments gave the same results.

It may be seen from Figures 1, 2, and 3, that the optimum temperatures for survival of the human red cell lie between 4° and 9° C.; with an optimal at about 7° C. ± 1°. This has been confirmed by the *in vivo* studies of Gibson, *et al* (2).

These experimental conditions do not represent the best we have found for human red cell survival, but they do illustrate the optimal temperature conditions for such survival.

Figure 4 presents data on survival of red cells

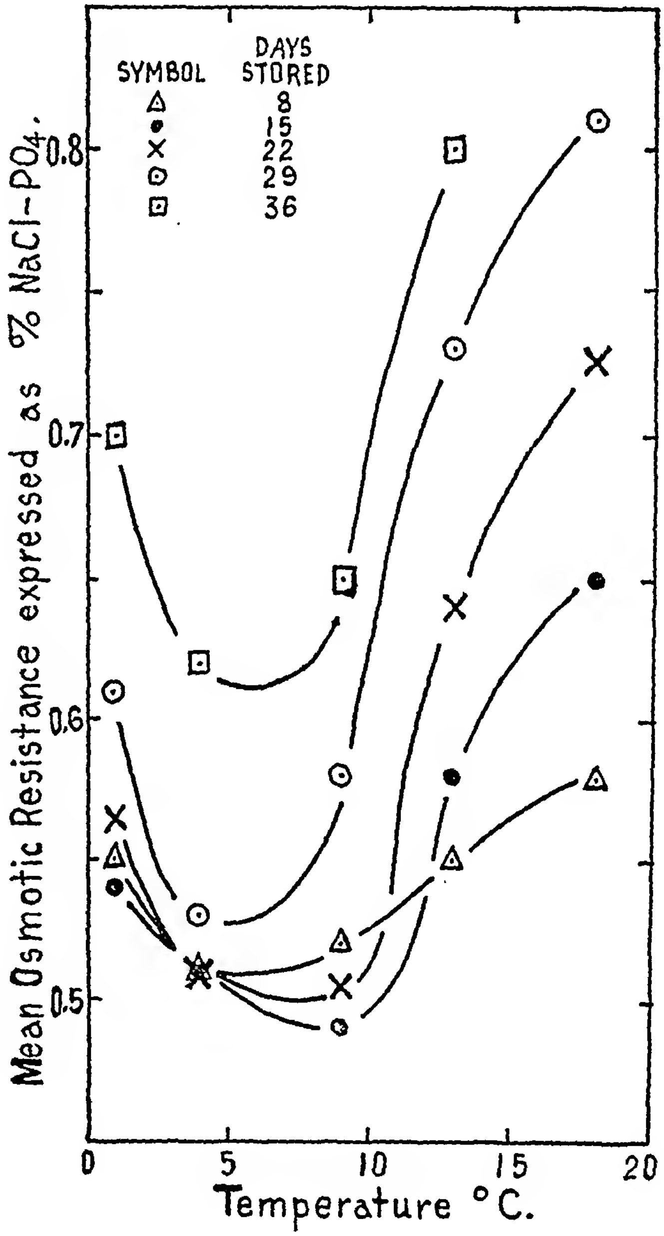

FIG. 2. EFFECT OF TEMPERATURE ON THE MEAN OSMOTIC RESISTANCE

under a variety of storage conditions at 2 different temperatures. One of these temperatures is in the optimal range (5° C.) while the other is well outside this range (23°). Figure 4 quite clearly shows that while temperature is a variable in the survival of stored red cells (one of prime importance as shown in Figures 1, 2, 3), and one whose effect is consistently in the same direction irrespective of large variations in the composition of the environment of stored red cells, nevertheless the effect of temperature may be overshadowed by the environmental composition. Curves a and b (Figure 4) contrast the rate of spontaneous hemolysis in the case of 12 collections of carefully defibrinated blood stored at 23° (a) and 6 collections stored at 5° (b) respectively. These blood samples had nothing else done to them except defibrination. It may be observed that the effect of temperature is present but its action is slight.

Compare these curves a and b with curves c and d (Figure 4). The latter 2 curves were obtained in 5 collections of blood according to the method of De Gowin, *et al.* (11) and stored at 23° and 5° respectively. In this case the effect of temperature is exceedingly striking and could have been deduced from the effect of temperature on the rate of penetration of glucose into human red cells, since the citrated bloods stored by De Gowin's method have been diluted to 40 per cent of their original concentration with an isosmotic solution of a compound that penetrates the red cells (5.4 per cent glucose). Such penetration of glucose leads to an abnormal increase in volume of the red cells (Figure 8).

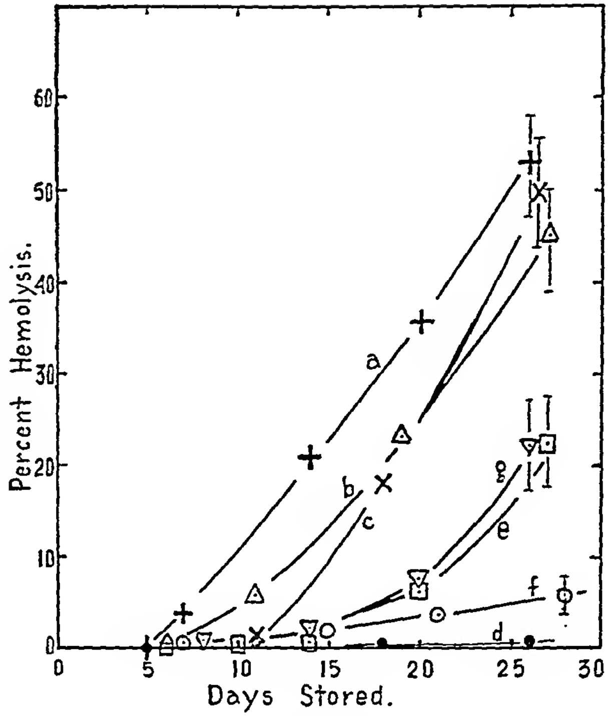

FIG. 4. SPONTANEOUS HEMOLYSIS AT 2 TEMPERATURES IN A VARIETY OF SOLUTIONS

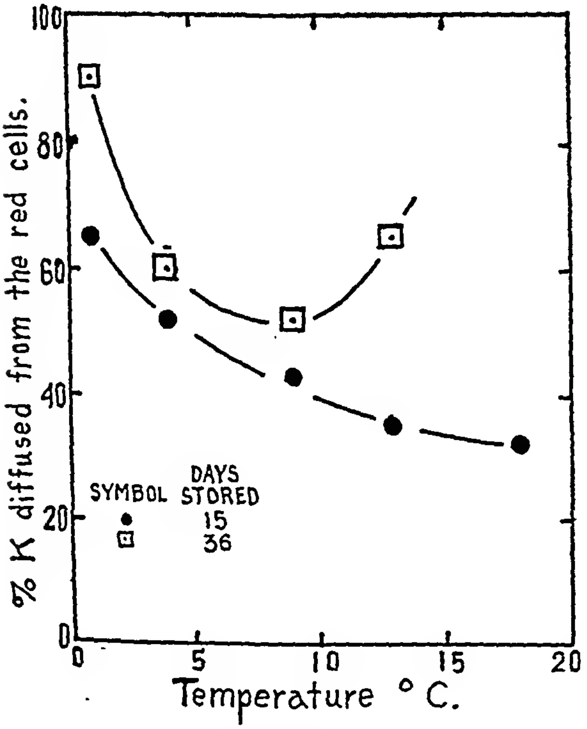

FIG. 3. INFLUENCE OF TEMPERATURE ON THE LOSS OF POTASSIUM FROM THE RED CELLS

One may also contrast curves e and f with a and b (Figure 4). The only difference other than temperature between the stored bloods represented in these 4 curves is the addition of sterile, solid glucose to give a final concentration of 0.5 per cent added glucose to the blood samples of curves e and f. These latter curves are the average for 6 and 9 individual blood collections. Comparison of curve e with f shows a very marked effect of temperature, over and above the strikingly better survival of the red cells to which glucose has been added.

Spontaneous hemolysis with time of storage for 90 parts of blood collected into 10 parts of an isosmotic $Na_3C_6H_5O_7 \cdot 2\ H_2O$ (0.11 M) solution

is shown in curve g (Figure 4). The better, though still poor, preservation under these conditions as compared with defibrinated blood stored at 5° C. (curve b) is primarily associated with the dilution of the blood and not with any effect of the added citrate. This point will be taken up later under the following section on the effect of dilution.

Figure 4 is intended solely to illustrate further effects of temperature on spontaneous hemolysis. It is not intended, however, to indicate that any of the methods of preservation illustrated in it is better than any other when considered from the point of view of *in vivo* survival. The other *in vitro* criteria that have been used on all of the samples of stored blood in Figure 4 indicate that none of these methods of preservation would give satisfactory *in vivo* survival expectancy.

2. The degree of dilution and preservation.

A review of the extensive literature on blood preservation reveals that a great variety of dilutions of the whole blood have been used. This has varied from 10 per cent whole blood through a great many intermediate degrees of dilution up to undiluted blood. In most cases in the literature, the use of a particular dilution, whether 10, 40, 50, 80, 90 per cent of whole blood or whole blood itself, has been purely arbitrary with no supporting data for a particular dilution. Further, the tonicity and glucose content of the dilution medium have varied along with the degree of dilution. The work described below was an attempt to correlate the survival of red cells with the degree of dilution of the stored blood.

In addition to the immediate problem of the

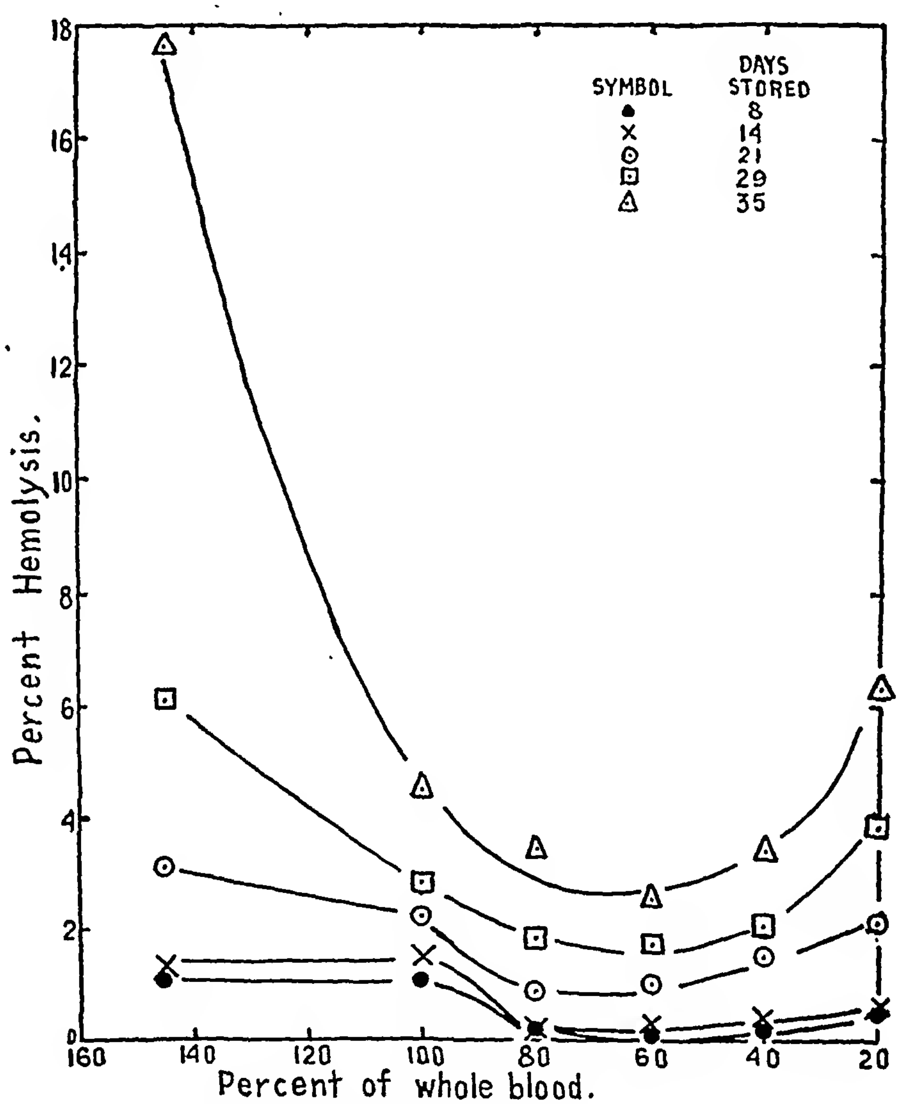

Fig. 5. Dilution of Whole Blood with Plasma and the Spontaneous Hemolysis During Storage

degree of dilution of stored blood on the survival of the red cells, there is a practical aspect of this problem in relation to transfusion. If it is necessary to increase the available hemoglobin of a patient, minimum dilution of the blood would be advisable. On the other hand the transfusion may also be used to combat dehydration, to supply glucose or salts, or plasma proteins, in which event high dilution of the red cells might be desirable. The answer to this must hinge upon a knowledge of what dilution is best for the survival of stored red cells.

An obvious perfect diluent might be plasma. We have run 5 experiments in which the donor's blood was diluted by varying amounts with its own plasma. A typical set of such data is given in Figure 5. The blood used in this experiment was collected by venipuncture into a bottle containing enough solid $Na_3C_6O_7 \cdot 2\ H_2O$ to give a final citrate content of 400 mgm. per 100 ml. of blood. A portion of the blood sample was transferred to sterile centrifuge tubes and citrated plasma obtained from it. This citrated plasma was then used to dilute the original blood by the amount indicated on the abscissa of Figure 5. Each of these diluted blood samples had 500 mgm. of glucose added to them and they were stored at the optimum temperature, 7° C. One of these blood samples was concentrated centrifugally and enough plasma removed to give a final concentration of 145 per cent relative to the whole blood concentration which was designated as 100 per cent. The blood of 3 other individuals had been stored and examined periodically in this way. Exactly similar results were obtained with only minor fluctuations in the absolute values.

The data contained in Figure 5 show quite clearly that the optimum dilution range for red cell preservation, with these conditions, lies between 80 per cent and 60 per cent of the whole blood concentration. It may also be noted that the optimum shifts from close to 80 per cent toward 60 per cent as the time of storage increases. On the average, blood which has been diluted to 70 per cent of its original concentration might be expected to give the best preservations of its red cells.

The question arises as to whether other types of diluents have an analogous effect. The data in Figure 6 illustrate this. Curve a (Figure 6) is

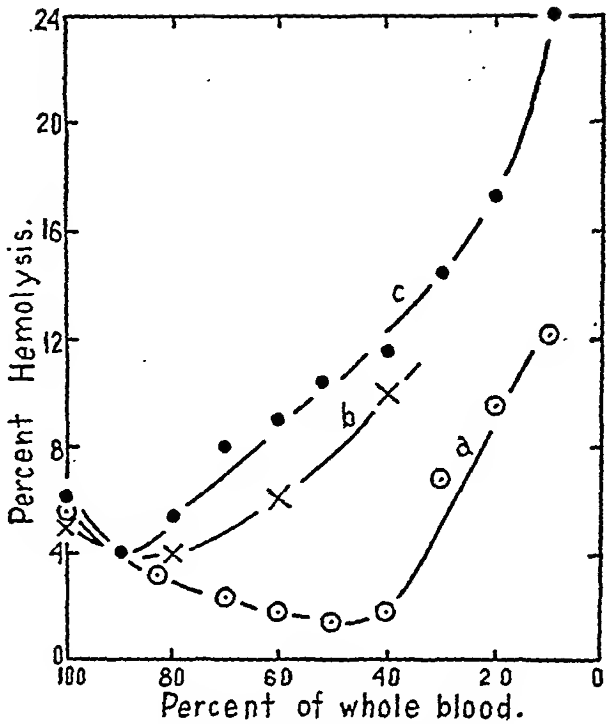

Fig. 6. Effect of Several Diluents on the Hemolysis Rate of Stored Blood

a plot of the degree of spontaneous hemolysis in blood stored for 48 days, at 7° C., under the following conditions. Five parts of blood were collected into 1 part of a 3.2 per cent solution of $Na_3C_6H_5O_7 \cdot 2\ H_2O$, cold (5° C.). A portion of this blood had 500 mgm. per cent (mgm. per 100 ml.) of glucose added, was stored at 7° C. and was examined at weekly intervals. This appears as 83.3 per cent of whole blood in curve a. Aliquots of the rest of this citrated blood were diluted with an isosmotic solution of glucose (5.4 per cent) to give the percentage concentrations of curve a. All were stored at 7° C. and examined at weekly intervals. The data in curve a (Figure 6) are those for the forty-eighth day of storage. They show that the optimum dilution lies between 60 and 40 per cent of the whole blood concentration and that a dilution which gives 40 per cent of the whole blood concentration is close to the borderline which may lead to rapid hemolysis. The latter is the preservative solution used by De Gowin, *et al* (11). The fact that survival of red cells in this solution is very poor when judged from the other *in vitro* criteria we have used has been previously mentioned under Figure 4, curve d.

Curve b (Figure 6) contains data for blood collected into solid citrate (see page 645) while curve c gives data for defibrinated blood. These blood samples were diluted with a sterile isotonic salt solution of the following composition: NaCl, 0.90 per cent; KCl, 0.042 per cent; $NaHCO_3$, 0.024 per cent; NaH_2PO_4, 0.05 per cent. They had 500 and 1,000 mgm. per cent of glucose added respectively and were stored at 7° C. The curves represent the degree of spontaneous hemolysis occurring at 35 and 21 days of storage respectively. The shapes of these curves and of curve a did not alter appreciably with time of storage, *i.e.* after the first week, though their position on the ordinate altered. It will be shown in section 5 that the difference in glucose concentration of these 2 samples does not account for the survival differences.

Curves b and c also show an optimum dilution and this occurs at about 80 and 90 per cent of whole blood. The effect is slight until blood concentrations of 60 per cent or lower are examined. It is obvious that dilutions of 60 per cent or greater are definitely deleterious when a balanced salt solution is used as the diluent. It is again noteworthy that defibrinated blood does not survive as well as citrated blood.

Figures 5 and 6 are convincing evidence of the fact that earlier experiments in the literature on blood preservation which failed to take account of the effect of dilution on survival of the red cells must be considered with respect to the actual dilution that was used. The foregoing experiments, combined with an analysis of data from a number of others, have led us to the conclusion that the survival time of stored red cells is increased when the blood is diluted. The data in Figures 5 and 6 would further indicate that for a variety of diluents a dilution consisting of 70 parts of blood and 30 parts of diluent (70 per cent whole blood) might be expected to give optimum survival.

Even this value of 70 per cent must be employed with caution, as the data of Figure 7 will show. In this case blood was collected into a minimum amount of citrate and diluted with a 6 per cent gelatine solution prepared in 0.9 per cent NaCl at pH 7.2.[2] The days of storage are indicated on the figure. Through 24 days of storage the survival was good at all dilutions and excellent at 40 per cent. At later periods of storage the survival was poor, but it is evident that the greater dilutions (40 and 20 per cent of whole blood concentration) gave better protection.

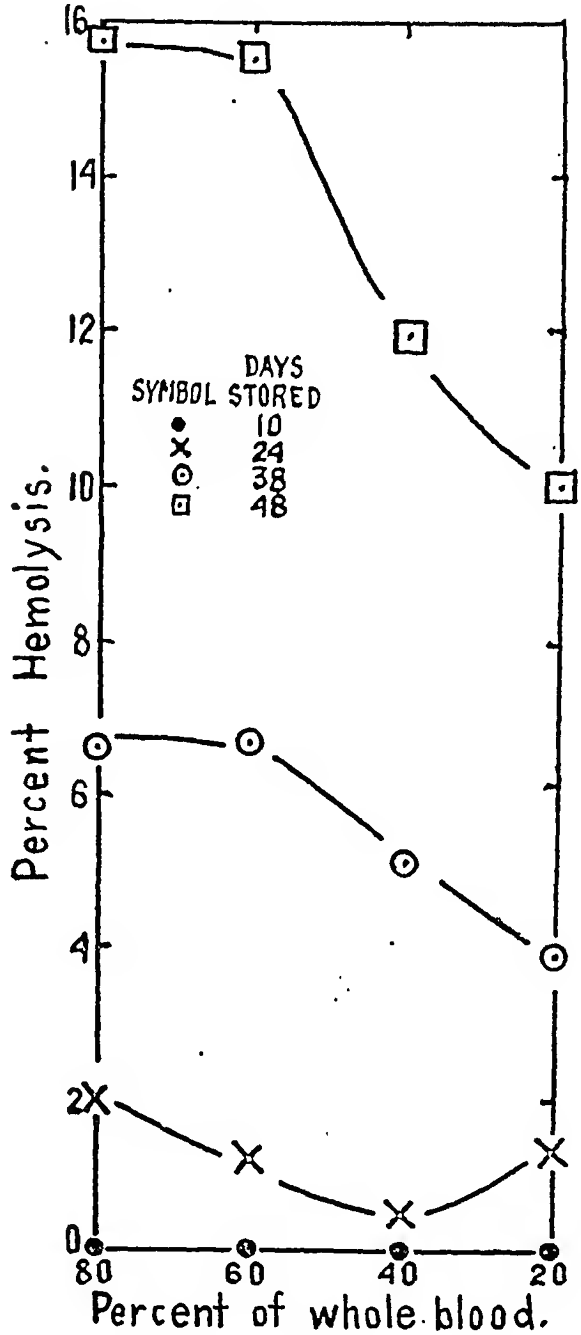

Fig. 7. Dilution with Gelatin

3. Tonicity, chemical composition and colloidal osmotic pressure of the diluent.

The diluent of historical interest is an isosmotic solution (5.4 per cent) of glucose (Rous and Turner [12]). They diluted whole blood as fol-

[2] This diluent was supplied through the courtesy of Dr. D. Tourtellotte, and is a product of Knox Gelatine Protein Products, Inc.

lows: 30 parts blood, 20 parts of 3.8 per cent $Na_3C_6H_5O_7 \cdot 5\ H_2O$ and 50 parts of a 5.4 per cent glucose solution. This has been used more recently at a 40 per cent concentration by De Gowin and his coworkers (11).

Those who have used this isosmotic glucose solution as a diluent for human red cells have ignored the fundamental fact that the human red cell is readily permeable to glucose. Thus, human blood collected by the methods of Rous and Turner or of De Gowin would be expected to show a marked increase in the volume of its red cells. That this is the case is shown in Figure 8, curve a, which expresses the relation between the red cell volume and the degree of dilution of the whole blood, under conditions where whole citrated blood was diluted with isosmotic glucose solution. The measurements given are for the seventh and forty-eighth days of storage at 7° C.

Contrasted with the very marked increase in volume of curve a are the much smaller red cell volume changes which occur during the storage of citrated blood which has been diluted with Ringer-Locke solution (calcium-free). The latter are represented in Figure 8, curves b and c, and are the data for the first and forty-ninth days of storage at 7° C. These diluted blood samples had 500 mgm. of added glucose.

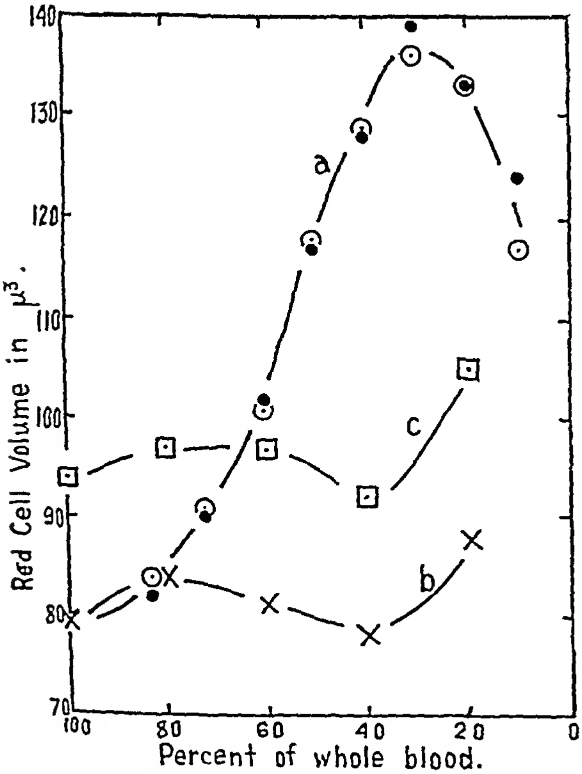

Fig. 8. Dilution and Red Cell Volume

The pronounced swelling of red cells diluted under the conditions of De Gowin or of Rous-Turner is the result of the penetration of the glucose. These red cells would continue to swell to the hemolytic volume were it not for the fact that they are being subjected to a shrinking effect brought on in part by the loss of chloride ions from the cells (Jacobs and Parpart [13]). An additional and more important factor in checking the swelling of human red cells diluted with isosmotic glucose solution is the rapid loss of potassium from these cells. Our data on potassium ion loss from the red cells stored under these conditions indicate that 50 per cent of the diffusion equilibrium is attained between the fifth and eighth days of storage, while the outward diffusion of potassium is complete after 10 to 20 days.

The balance between these several factors acting on the cell volume is a delicate one and needs only a slight change in environmental conditions (*e.g.* temperature rise) to cause a rapid rate of spontaneous hemolysis by the red cells of blood thus diluted, see curve c (Figure 4). Human blood stored under conditions where it is diluted to 40 or 30 per cent of its original concentration with an isosmotic glucose solution would be expected to show very poor *in vivo* survival of its red cells (Gibson [2]).

The question has been frequently raised as to whether it is important to maintain the normal colloidal osmotic pressure of the fluid surrounding the stored red cells. In an earlier section of this paper we have demonstrated that dilution of whole blood to 70 per cent of normal affords a better environment for the survival of the red cells. This was true irrespective of whether the diluent was (*1*) plasma, (*2*) a 6 per cent gelatine in isotonic NaCl solution, (*3*) a balanced salt solution, (*4*) an isosmotic glucose solution, or (*5*) an isotonic phosphate solution which when added to the blood established an initial pH of 7.1 (Figures 5, 7, 6, and 9).

Diluents (*1*) and (*2*) above had normal colloidal osmotic pressure while the other 3 diluents reduced the colloidal osmotic pressure of the plasma of the stored blood to 70 per cent of normal. The

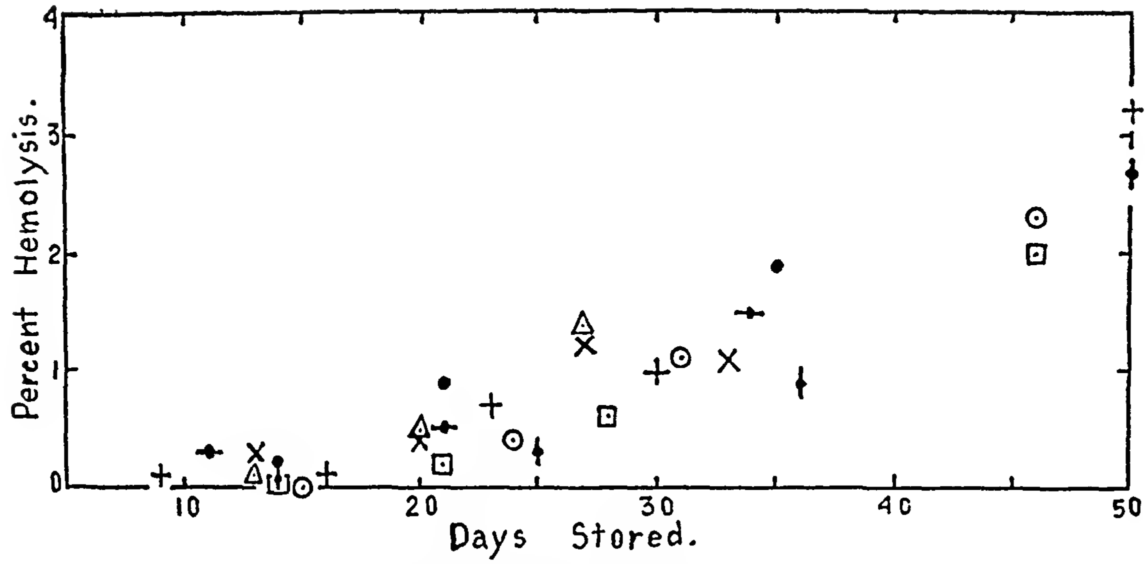

FIG. 9. COMPARISON OF DATA ON PHOSPHATE DILUTED BLOODS

rate of spontaneous hemolysis in these various diluents, however, did not differ greatly and what differences there are certainly cannot be ascribed to this factor. In fact, the survival of the red cells in stored blood diluted to 70 per cent of whole blood concentration with the phosphate solution (Figure 9) is much better than that in plasma or gelatine solution judged from our *in vitro* data and the *in vivo* data of Gibson (2).

It should be pointed out that the differences that do exist are outside the limits of variation of individual blood samples. This is also illustrated by Figure 9, where the degree of spontaneous hemolysis is plotted against the days of storage. The 8 individual blood samples represented in Figure 9 were from data taken at random over a period of 1½ years on citrated human blood samples stored at 7° C. and diluted as follows: 70 parts of whole citrated blood (4 mgm. $Na_3C_6H_5O_7 \cdot 2\,H_2O$ per ml. of blood) and 30 parts of a solution containing 60 volumes of 0.11 M $Na_2\,HPO_4$ and 40 volumes of 0.11 M NaH_2PO_4. The initial pH of blood thus diluted is 7.1 and during 50 days of storage drops to pH 6.7.

Another series of tests on the influence of the colloidal osmotic pressure of the plasma on red cell survival were run on 4 different samples of human blood. The blood samples were collected into citrate (in amount indicated above), divided into 2 equal volumes, transferred to sterile centrifuge tubes, spun, and the major portion of the plasma removed aseptically. The plasma of 1 aliquot of cells was exactly replaced by a solution recommended by Gibson, viz.:

$Na_3C_6H_5O_7 \cdot 2H_2O$	480 mgm.
Glucose	1,080 mgm.
NaCl	590 mgm.
Conc. HCl (Sp. G. 1.19)	0.25 ml.

made up to 100 ml. with pyrogen-free water.

The other aliquot had its plasma replaced by the same solution except that 3 grams of plasma protein- lipid Fraction IV–3, 4 Run S301, Cohn (14), were added to the 100 ml. of solution before replacing the plasma.

In the case of all 4 blood samples thus treated there was good survival over 45 days storage, but the difference slightly favored the solution without the added Fraction IV. In brief, the colloidal osmotic pressure of the medium surrounding human red cells does not appear to be a factor in their *in vitro* survival.

4. pH range and optimum, buffering.

The influence of pH on the survival of stored red cells has not been consistently investigated. Recently Loutit (1) has gathered together data on a number of samples of blood stored in acid citrate-dextrose that indicate that blood diluted with this solution and having an initial pH of 7.1 to 7.3 shows a better survival of its red cells.

In a report, dated October, 1943, to the Blood Substitutes Committee of the Committee on Medical Research we showed the very marked influence of pH on the survival of the red cells of stored human blood. Our studies of the influence of pH

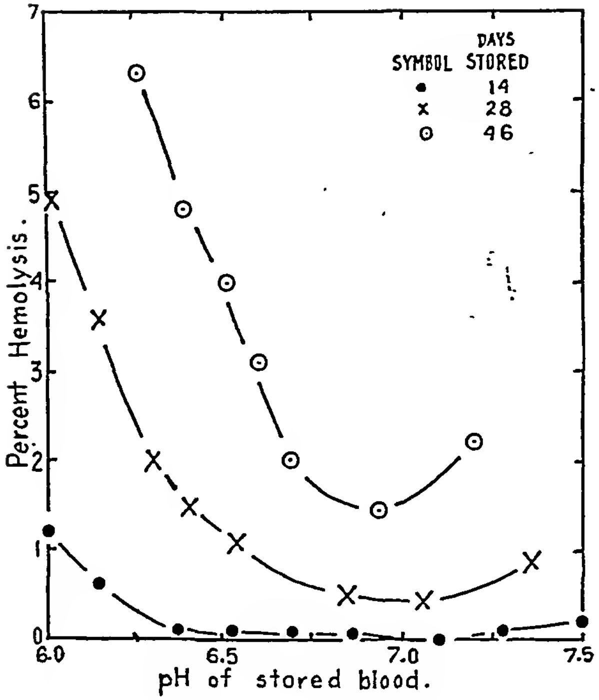

FIG. 10. INFLUENCE OF pH ON HEMOLYSIS RATE OF STORED BLOOD

on *in vitro* survival have been carried out under conditions where a minimum shift in the pH of stored blood was attained during long periods of storage. The citrate buffer system has no buffer capacity over the pH range for maximum survival of stored red cells (Figure 10). The rate of glycolysis of blood and the rapid formation of lactic acid, even at the optimum temperature (7° C.), is so high that over long storage periods blood samples will show marked decreases in pH unless adequately buffered.

We, therefore, turned to the phosphate buffer system and studied survival of the red cells at various hydrogen ion concentrations. These blood samples were followed over long time periods under conditions where a minimum decrease in pH with time of storage occurred. Citrated blood was diluted with isotonic (0.11 M) phosphate buffer solutions in the proportion of 30 parts of buffer to 70 parts of whole blood. In all cases these stored bloods had 0.5 per cent added glucose and storage was at 7° C. Thus, a number of blood samples having an initial pH between 6.0 and 7.5 were prepared and red cell survival determined at frequent intervals. Seven individual blood samples were run in this manner; the typical result of the effect of pH is shown in Figure 10 where the per cent of spontaneous hemolysis is plotted against the pH of the blood samples at the fourteenth, twenty-eighth, and forty-sixth days of storage.

Figure 10 demonstrates that the optimum pH for prolonged storage of human blood lies between pH 6.7 and 7.0. It may also be noted that during the first 2 weeks of storage there is slight effect of pH over the range pH 6.5 to 7.5. Longer storage periods, however, show a marked influence of pH. Thus storage for 4 and 7 weeks clearly requires a pH between 6.7 and 7.0 if optimum preservation of the red cells is to be attained.

Another striking action of pH on the survival of the red cells of stored blood is on the ability of the red cells to retain their normally high potassium content. This effect is illustrated in Figure 11, where the per cent of the original amount of potassium in the red cell which has diffused out of the cell at various times of storage is plotted against the pH of storage. In this experiment the blood was buffered by phosphate solutions as described above and 0.5 per cent glucose added and stored at 7° C.

Figure 11 illustrates that human red cells stored at a pH between 6.7 and 7.0 will lose their potas-

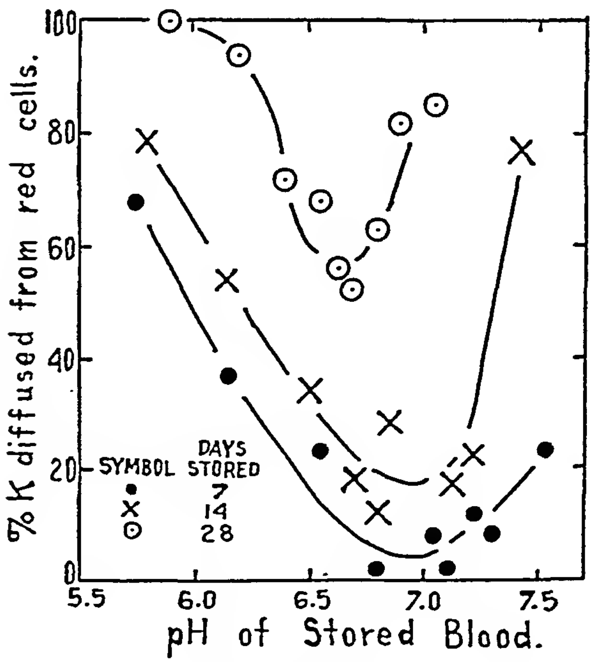

FIG. 11. EFFECT OF pH ON THE RATE OF LOSS OF POTASSIUM

sium at a much less rapid rate than at a pH higher or lower than this optimum range. Even after 4 weeks of storage at pH 6.8 the potassium of the red cells has diffused to only 60 per cent of equilibrium.

The studies thus far dealt with have employed the phosphate system. The results outlined are due primarily to the pH and not to specific action of the phosphate itself. This has been established by experiments involving storage of human blood in maleic, in maleic-succinate, and in glycyl-glycine buffer systems, under conditions otherwise identical for the phosphate system. The data obtained for these 3 systems as concern the optimum pH range for red cell survival are the same as that for the phosphate system, though the absolute magnitude at the optimum pH for cell survival does vary with the buffer used (Table I).

TABLE I

Comparison of the per cent of spontaneous hemolysis in human blood after 4 weeks' storage at 7° C. in the presence of different buffer systems

Each blood sample was composed of a mixture of 30 parts isotonic buffer solution and 70 parts whole (citrated) blood. The initial pH of each was 7.10 to 7.20 and the pH on the fourth week of storage is given. Each blood sample had 0.5 per cent glucose added.

Buffer system	pH after 4 weeks' storage	Spontaneous hemolysis after 4 weeks	Original glucose utilized by fourth week
		per cent	*per cent*
NaH_2PO_4/Na_2HPO_4	6.82	0.5	20
Glycyl-glycine/Na glycyl-glycine	6.75	0.7	18
Maleic-succinic acids/Na maleate-Na succinate	6.95	7.0	13
Maleic acids/Na maleate	6.82	15.0	0
None	6.94	1.8	36

In this respect, Table I shows that the phosphate system was better than the other buffers. It is thus certain that in addition to the pH effect on survival the phosphate itself markedly increases survival. The glycyl-glycine buffer is also exceptionally effective in prolonging red cell survival of stored blood (Table I).

Table I also shows that red cell survival in the maleic and maleic-succinate systems is poor even at the optimum pH. This effect, however, is probably associated with the low or completely inhibited glycolytic rate in the presence of these latter buffer systems (Table I).

Citrated blood, diluted to 70 per cent of whole blood with its own plasma and with 0.5 per cent added glucose, stored 4 weeks at 7° C., but with no buffer system added is included in Table I. It may be seen that while the degree of spontaneous hemolysis is low at this time, it is, however, considerably greater than in the blood with phosphate or glycyl-glycine buffer system (see also Figure 5).

From the foregoing it would appear that the phosphate buffer solutions which have on mixing with blood an initial pH between 7.1 and 7.3 and a pH after 4 weeks of storage at about 6.9 (with 0.5 per cent added glucose and storage at 7° C.) are exceptionally fine for increasing the survival period of the red cells of stored blood of man. The question remains as to what might be the minimal concentration of phosphate buffer to add to blood for optimal or good preservation of the red cells.

To answer this question, whole citrated human blood was diluted with varying amounts of phosphate buffer (Table II). The degree of dilution

TABLE II

Blood sample	Citrated blood	Phosphate buffer mixture	Balanced salt solution	Added glucose	pH 0 days storage	pH 50 days storage
no.	*ml.*	*ml.*	*ml.*	*mgm.*		
397	70	30	0	500	7.29	6.74
398	70	20	10	500	7.35	6.78
399	70	10	20	500	7.48	6.86
400	70	3	27	500	7.54	6.90
401	70	0	30	500	7.59	7.03

(70 per cent whole blood) was maintained constant (Table II), by using a calcium and phosphate-free Ringer-Dale solution. The glucose added to each blood sample was 0.5 per cent and all bloods were stored at 7° C. The data in Table II are expressed in terms of 100 ml. of blood plus diluent. The phosphate buffer mixture used consisted of 60 parts of 0.11 M Na_2HPO_4 and 40 parts of 0.11 M NaH_2PO_4.

The degree of spontaneous hemolysis which occurred in these stored samples after 14, 36, and 50 days of storage is illustrated in Figure 12. The "mean osmotic resistance" initially and at the same storage times is given in Figure 13.

As noted before, the presence or absence of the phosphate buffer has little or no effect during the first 2 weeks of storage (Figure 12). However,

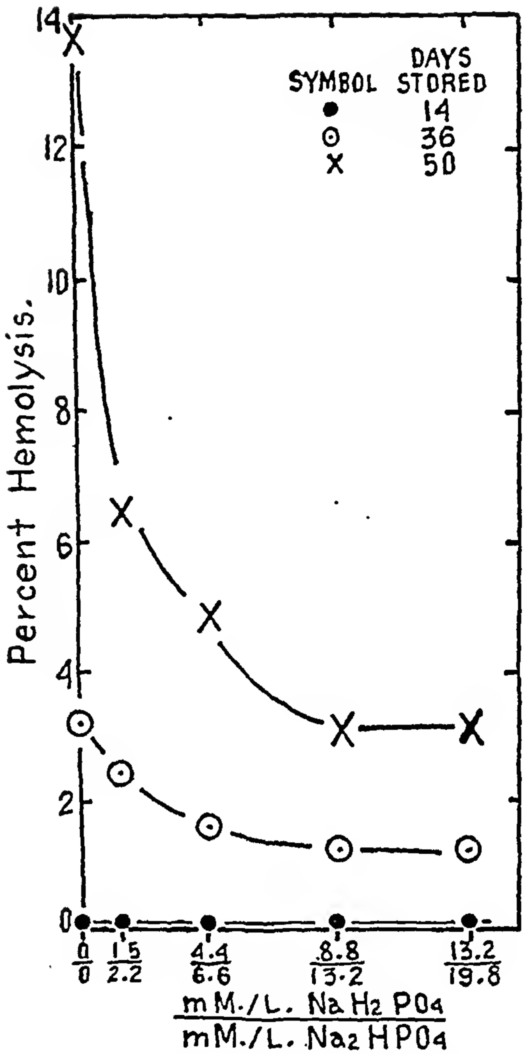

FIG. 12. EFFECT OF PHOSPHATE CONCENTRATION ON SPONTANEOUS HEMOLYSIS

when one examines the mean osmotic resistance curves (Figure 13) one observes that even after 2 weeks of storage the bloods stored with no, or a small amount of, phosphate buffer would be poor risks in transfusion and this becomes more striking on the fifth and seventh weeks of storage.

Blood stored with 0.0044 M NaH_2PO_4 and 0.0066 M Na_2HPO_4 per liter of blood and with 0.5 per cent added glucose, 30 per cent dilution and at 7° C., has its red cells well preserved over 5 weeks of storage. Blood stored under these conditions and injected in 500 ml. amounts would increase the normal plasma phosphate level by only 40 per cent.

5. Optimal Glucose Content.

Glucose added to stored blood increases the red cell survival. This is a fact upon which all experiments on blood preservation have concurred. No other sugar has been observed to have a comparable effect.

Surprisingly no one has investigated the effect of varying the glucose concentration while keeping other possible variables as constant as possible. We therefore performed a series of experiments on the effect of glucose concentration on red cell survival of stored human blood. Several series compared the survival in whole blood to which varying amounts of glucose were added. Blood was collected directly into solid citrate in the proportion of 400 mgm. $Na_3C_6H_5O_7 \cdot 2\ H_2O$ to each 100 ml. of blood. To aliquots of this citrated blood, amounts of glucose varying from 0 to 6 per cent were added and the survival followed over a 7-week period. The glucose was added on the basis

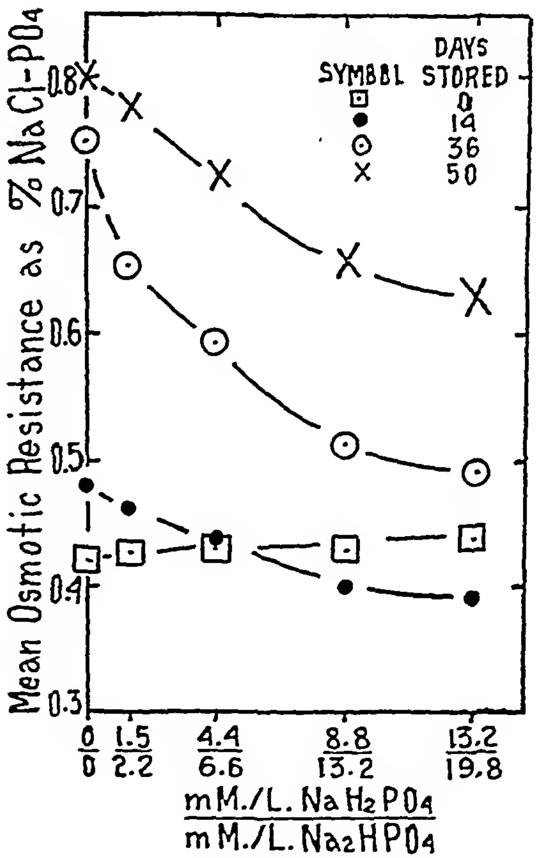

FIG. 13. EFFECT OF PHOSPHATE CONCENTRATION ON MEAN OSMOTIC RESISTANCE

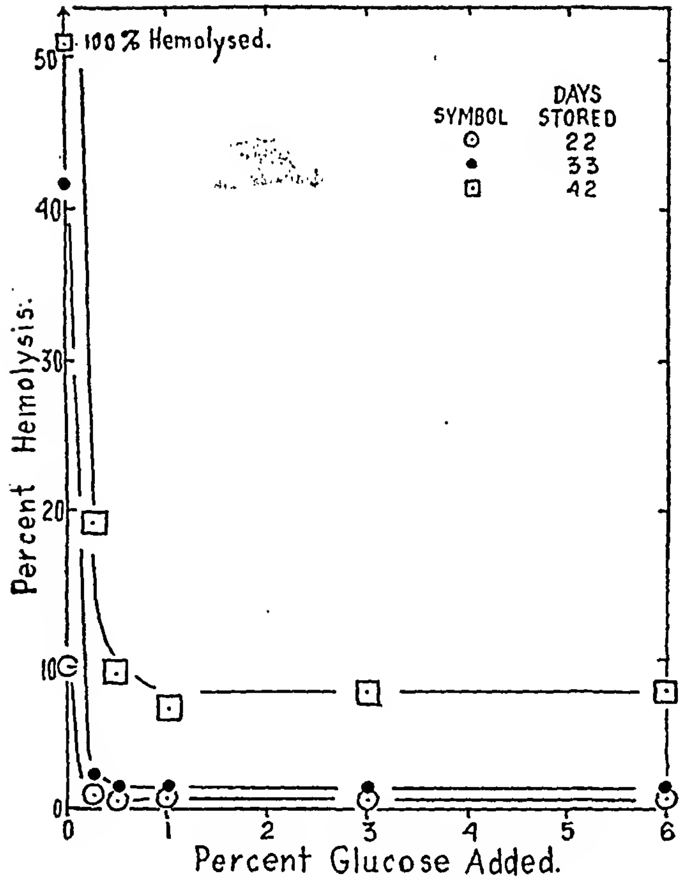

FIG. 14. OPTIMAL GLUCOSE CONCENTRATION FOR STORAGE

of grams of glucose per 100 ml. of stored blood, *e.g.* 1 per cent means 1 gram per 100 ml. blood. All bloods were stored at 7° C. The results of such experiments are illustrated in Figures 14, 15, and 16.

Data on the effect of added glucose on the degree of spontaneous hemolysis occurring during storage are given in Figure 14. The data in this figure show quite clearly that even a low total concentration (0.25 per cent) of added glucose aids in the red cell survival for 33 days of storage when compared with the cells of blood stored with no added glucose. From 33 to 42 days of storage, however, this low concentration becomes insufficient. It may also be noted in Figure 14 that amounts of glucose, including 0.5 per cent and greater, increase to a considerable degree the red cell survival although there is no cumulative effect of the greater amounts of glucose. It must be emphasized that these experiments were designed to vary only 1 factor, namely, glucose concentration. This, of course, precluded the possibility of studying survival under the optimal conditions of dilution and buffering against pH change. The results as far as the minimal effective amount of glucose to add to stored blood, viz. 0.5 per cent, are quite conclusive. Glucose penetrates the human red cell and, hence, its osmotic effects may be neglected under the conditions of these experiments.

Completely analagous results have been obtained on citrated blood diluted to 70 per cent with phosphate buffer at the optimal pH and stored with and without added glucose. After 42 days of storage these blood samples showed 8 per cent spontaneous hemolysis when no glucose was added and 1.8 per cent in the presence of 0.5 per cent added glucose. These data contrast only in the magnitude of the hemolysis with those for 42 days' storage in Figure 14 but are parallel as to the action of glucose. They show again the importance of dilution and buffering. In fact, blood stored under these latter conditions is in better state for

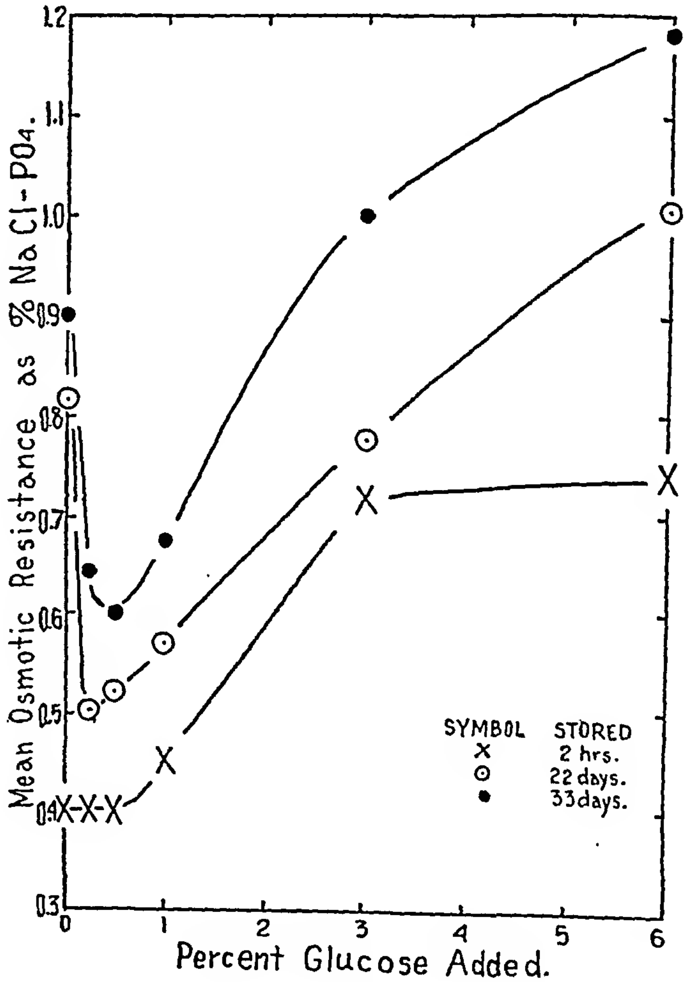

FIG. 15. EFFECT OF ADDED GLUCOSE CONCENTRATION ON THE MEAN OSMOTIC RESISTANCE

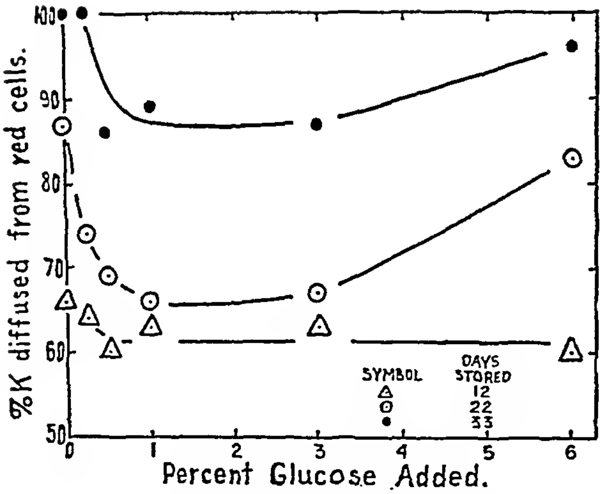

FIG. 16. EFFECT OF ADDED GLUCOSE ON THE LOSS OF POTASSIUM

transfusion after 42 days of storage than undiluted, unbuffered blood was at 22 days, regardless of added glucose.

Figure 15 brings out the noteworthy fact that high concentrations of added glucose, 1 per cent and higher, lead to a rapid decrease in the osmotic resistance of the stored red cells. These experimental observations are obviously the resultant of the effect of the increased concentration of glucose in the cells stored with this high amount of added glucose and the consequent swelling this would produce in the hypotonic saline solution in which the osmotic resistance was measured. They do give point to the fact, however, that such red cells when transfused would undergo rapid osmotic hemolysis, thus rendering them useless for such purposes. This danger point lies between 1 and 3 per cent added glucose (Figure 15).

The slight, but noticeable effect of added glucose on the retention of potassium by the stored red cells is illustrated in Figure 16 and may be contrasted with the good retention of potassium when the blood is diluted and buffered to the optimum pH (Figure 11).

We have studied the preservative action of a number of sugars in comparison with glucose. In all of these studies the particular sugar was compared with identical conditions for survival with added glucose. The sugars used were xylose, lactose, fructose, maltose, sucrose. None of these sugars gave as good survival of the red cells of stored blood as compared with cells stored with added glucose. In all cases except xylose the red cell survival was better than under parallel experiments where no sugar was added. Xylose gave no better survival than control blood with no added sugar.

SUMMARY

An analysis has been made of a number of factors concerned in prolonging the life of the red cells of stored human blood.

Blood collected and stored under the following conditions has been found to give excellent survival of its red cells over a period of at least 6 weeks: Seventy parts of blood collected into 10 parts of 4 per cent $Na_3C_6H_5O_7 \cdot 2\ H_2O$ and 20 parts of a phosphate buffer solution (composed of 60 volumes 0.11 M Na_2HPO_4 and 40 volumes of 0.11 M NaH_2PO_4); 500 mgm. per cent of glucose is added and the blood is stored at 7° C.

Blood thus stored will be under the optimal conditions of (*1*) pH (initially *7.2* and 6.8 after 6 weeks), (*2*) good buffering, (*3*) dilution (*70* per cent), (*4*) glucose concentration (500 *mgm.* per cent), (*5*) temperature (*7°* C.).

BIBLIOGRAPHY

1. Loutit, J. F., Factors influencing the preservation of stored red cells. J. Path. & Bact., 1945, 57, 325.
2. Gibson, J. G., 2nd, Evans, R. D., Aub, J. C., Sack, T., and Peacock, W. C., The post-transfusion survival of preserved human erythrocytes stored as whole blood or in resuspension, after removal of plasma, by means of two isotopes of radioactive iron. J. Clin. Invest., 1947, 26, 715.
3. Chase, A. M., Lorenz, P. B., Parpart, A. K., and Gregg, J. R., A method for simultaneous determination of methemoglobin-oxyhemoglobin ratio and per cent hemolysis in stored blood. J. Lab. & Clin. Med., 1944, 29, 99.
4. Parpart, A. K., Lorenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, A. M., The osmotic resistance (fragility) of human red cells. J. Clin. Invest., 1947, 26, 636.
5. Weichselbaum, T. E., Somogyi, M., and Rusk, H .A., A method for the determination of small amounts of potassium. J. Biol. Chem., 1940, 132, 343.
6. Hoffman, W. S., A rapid photoelectric method for the determination of glucose in blood and urine. J. Biol. Chem., 1937, 120, 51.
7. Barker, S. B., and Summerson, W. H., The colorimetric determination of lactic acid in biological material. J. Biol. Chem., 1941, 138, 535.
8. Parpart, A. K., and Ballentine, R., Hematocrit determination of relative cell volume. Science, 1943, 98, 545.

9. Parpart, A. K., Is osmotic hemolysis an all-or-none phenomenon? Biol. Bull., 1931, 61, 500.
10. Parpart, A. K., The permeability of the mamalian erythrocyte to deuterium oxide (heavy water). J. Cell. & Comp. Physiol., 1935, 7, 153.
11. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. J. A. M. A., 1940, 114, 850.
12. Rous, P., and Turner, J. R., The preservation of living red blood cells *in vitro*. J. Exper. Med., 1916, 23, 219.
13. Jacobs, M. H., and Parpart, A. K., Osmotic properties of the erythrocyte. Biol. Bull., 1933, 65, 512.
14. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, S. H., Jr., Chemical, clinical and immunological studies on the products of human plasma fractionation. J. Clin. Invest., 1944, 23, 417.

ESTIMATION OF CELL SURVIVAL AFTER TRANSFUSION BY SELECTIVE AGGLUTINATION

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Selective agglutination, as a means of estimating the number of donor's erythrocytes in the circulation of a recipient after transfusion, was used first by Ashby (1) in 1919. In the procedure, group-O blood was administered to a subject of group A (or B), and in blood specimens taken periodically thereafter, the number of surviving donor-cells was estimated by selectively agglutinating those of the recipient with anti-A (or anti-B) serum, and counting the unagglutinated group-O cells in a hemocytometer. Wiener and Schaefer (2, 3), in 1939, obtained the same result by using the M and N blood types. More recently still Wiener (16), and Mollison (4) have extended the technique to the Rh blood types using Rh-negative donors and Rh-positive recipients with anti-Rh as the agglutinating serum. Wiener and Peters (5), and Mollison and Young (6) have demonstrated the adaptability of the method by following the survival of 2 donors' cells simultaneously in the same recipient. The results obtained by these techniques are in good agreement, and show that if fresh blood is used for transfusion the donor's erythrocytes disappear from the recipient's circulation at the rate of about 1 per cent per day, which is the normal rate of red cell destruction. In the past 8 years the method has proved to be of great value in studies on blood preservation, particularly in testing the effects of various methods of preservation on the viability of erythrocytes during storage. From the practical standpoint, the O and A blood groups offer the advantage that they occur frequently in the population and that highly potent anti-A serum is readily obtainable.

The original agglutination procedure of Ashby has been modified in various laboratories with regard to the amounts of cell suspension and antiserum used, temperature conditions, and technique. Numerous workers (1, 3, 7, 8, 9, 10) perform the reaction in small serological test tubes with or without agitation. Maizels and Paterson (11) use small beakers, and Loutit (12), small bottles with a relatively small amount of antiserum. Thalhimer (13) has reduced the number of manipulations to a minimum by carrying out the reaction in a red-cell pipette. The present authors have modified their former method by using light centrifugation to accelerate agglutination. This treatment is also a feature of the method of Dacie and Mollison except in the use of anti-M and anti-N sera (14) which are said to give some non-specific agglutination with centrifugation. All these procedures are based on extensive experimentation and, in the hands of experienced workers, give comparable results.

In determining the number of the donor's erythrocytes in the recipient's circulation by selective agglutination, it is necessary beforehand to know the recipient's "free" or "unagglutinable" cell count. In other words since complete agglutination of erythrocytes is impossible by ordinary methods the number of unagglutinable cells per mm.^{3} must be ascertained prior to transfusion. The number of donor's cells in the post-transfusion samples may be determined by subtracting the recipient's free-cell count from the total number per mm.^{3} Experience has shown that the free-cell count of the individual is fairly constant with a given potent antiserum, but it may vary with different antisera depending largely on the agglutinin potency, or titre. Authorities are agreed that strong antiserum is essential for accurate results. The consensus is that an antiserum for selective agglutination tests should be specific for the recipient's red cells and should give a free-cell count not exceeding 50,000 per mm.^{3} of blood.

From time to time the reliability of the selective agglutination method has been queried. The various criticisms and their refutation have been discussed by Maizels and Paterson (11) and by Mollison and Young (15).

The study reported in the present paper arose from certain anomalies observed in an earlier investigation on red cell survival carried on during

1940 to 1943. At that time we were using the method of Wiener and Schaefer (3) with anti-M and anti-N sera prepared in our laboratory according to the methods of Wiener (16) and of Davidsohn and Rosenfeld (17). The antisera, which were prepared aseptically, were potent and highly specific. Prior to use, the serum for a complete experiment was given an additional absorption with a light suspension of saline-washed red cells of the prospective recipient to ensure the absence of nonspecific agglutinins. Frequently, the serum, if strong, was diluted further before dispensing it into small serological tubes. The latter then were stored at 0° C. These antisera were of high avidity, producing visible and compact agglutination within a few seconds. The free-cell count usually was less than 30,000 per mm.^{3} but up to 50,000 was considered satisfactory.

The above mentioned anomalies in our cell-survival results were periodic increases in the number of unagglutinable cells, a condition which should not have existed provided that the recipient's free-cell count remained constant and the transfused cells were being destroyed continuously. That the increases were not attributable to errors in counting or to alteration in the antiserum was ruled out by repetition of the counts with several lots of the antiserum. Some of the tests were repeated as many as 12 times with consistent results; others were checked by both authors. In several instances the deviations in the survival counts followed a steady course over a period of 2 or 3 weeks and exhibited a periodicity which was inexplicable. That some erythrocytes were being sequestered from the circulation and released again periodically was hardly tenable. As other investigators (3, 15) in similar studies had not encountered the same type or degree of irregularity, it was decided to investigate further into the cause of the phenomenon. Attention was directed first to ascertaining the constancy of the free-cell count and secondly to the influence of the titre and avidity of the antiserum on the completeness of agglutination.

Discussion of the more theoretical aspects of agglutination will be reserved for a later publication. For a survey of the literature relevant to the present study the reader is referred to recent reviews (16, 18, 19) and to papers by Eagle (20, 21), Duncan (22) and Kendall (23).

Whatever the mechanism of agglutination may be, authorities are agreed that the phenomenon involves 2 main stages, namely; (*1*) the combination of the antigen (agglutinogen) with the antibody (agglutinin) and (*2*) aggregation of the sensitized cells. Sensitization is known to occur very rapidly while aggregation is a slower process. Eagle (21) and others have shown that mechanical agitation accelerates the rate of agglutination by increasing the incidence of collision of the cells. The process is very rapid at first but decreases logarithmically with time. It may be brought about more rapidly and firmly with the aid of centrifugation. Thus the titre of an antiserum, when determined with centrifugation is from 6 to 10 times that without the treatment. Up to a point agglutination increases with the concentration of agglutinin. Very potent antisera may not produce agglutination or may not give a maximum reaction until diluted. This upper zone of inhibition is usually called the "prozone" to distinguish it from the lower zone, the "postzone" where aggregation of cells is diminished due to dilution of the antibody. The mechanism of prozone inhibition is not fully understood although considerable light has been thrown upon the subject by the studies of Jones and Orcutt (24).

The inadequacy of the ordinary titre method for characterizing the potency of an antiserum has been stressed by numerous authors. Obviously the titre, expressed as a number, merely gives a rough and comparative idea of the agglutinin concentration of the original material. For the purposes of the clinical laboratory, however, the method is valuable particularly for determining the point of optimal proportion of antigen and antibody, *i.e.*, the dilution which produces most rapid agglutination. With moderately strong or weak serum, the maximum rate of agglutination is obtained with the undiluted material. As erythrocytes of different individuals of the same blood group vary considerably in properties, the titre of a given serum and the concentrations of optimum proportion may vary with the blood tested.

In the present study the authors have sought an answer to the following questions:

(*1*) Can the free-cell count be abolished by careful adjustment of the antiserum concentration and by control of conditions of the reaction?

(2) What is the normal variation in the free-cell count?

(3) To what degree is incomplete agglutination of red cells influenced by the following factors:

(a) Too rapid agglutination and insufficient time for completion of the reaction.

(b) Insufficient antibody.

(c) Under-sensitization of cells due to deficiency in antigen content, or to age.

EXPERIMENTAL

In 1944 the present authors modified their agglutination technique by introducing centrifugation, thus reducing the time of the procedure to about one-third. Also, with the increased availability of highly potent anti-A and anti-B sera a change was made to the O, A, and B blood groups in preference to the M and N types.

METHODS

1. *Selection of antiserum*

In selecting an antiserum for an experiment an ordinary titre series is set up for each serum to be tested using the saline-washed red cells of the prospective recipient. With the aid of a microscope the particular dilution in the series which gives the most complete agglutination is ascertained. This concentration may not be the one which produces the most rapid agglutination or the most compact cell sediment. Having selected the optimum serum concentration for completeness of reaction, a larger quantity of the particular dilution is prepared and dispensed aseptically into small tubes which then are stoppered and stored at 0° C. Prior to use, each tube is permitted to come to room temperature and is inverted several times to ensure uniformity of the contents.

Very potent anti-A or anti-B sera, particularly those prepared by concentrating the globulin fraction, frequently develop a surface precipitate of lipid when stored at 0° C. or after having been frozen. Unless removed, this material may prove a source of much annoyance and confusion during cell counting since many of the particles are about the size of the erythrocytes. One of the authors (D. E. O.) has found that the lipid can easily be removed by keeping the serum in a centrifuge tube in the cold for a few days and then cautiously overlaying the surface with physiological saline followed by centrifugation at high speed. If the saline is applied slowly with a fine capillary pipette, no mixing with the serum will occur and, after centrifuging, the saline layer, on the surface of which the lipid will have collected, can be removed by aspiration through a capillary tube.

2. *Collection of blood samples*

Prior to transfusion, and periodically thereafter, blood samples are taken from an antecubital vein of the recipient. Approximately 10 ml. are delivered into a graduated centrifuge tube containing 1 ml. of sterile sodium citrate solution (3.2 per cent). The tube is stoppered and inverted gently several times. By emptying the syringe against the wall of the tube and by using gentle mixing methods excessive frothing of the sample can be avoided. Foam may be collapsed by touching it with a fine wire or needle the mere tip of which has been dipped into caprylic alcohol. The total volume of the specimen is recorded to obtain the dilution factor. The hemoglobin concentration of the specimen is determined with a photoelectric colorimeter, and the hematocrit, using the Wintrobe tube. A total red-cell count is performed on the pretransfusion specimen.

3. *Agglutination procedure*

Blood samples, if refrigerated, are permitted to come to room temperature. The red cells are suspended by gently and repeatedly inverting the tube for about 1 minute. Another light mixing is performed immediately before pipetting.

Blood is drawn up to the 0.5 mark in a white-cell pipette which is then filled to the 11 mark with sodium chloride solution (0.9 per cent). After the pipette is shaken for a few seconds, it is emptied into a serological tube (10×75 mm.).

The tube is shaken a few times, and the cell suspension is drawn up to the 1.0 mark in a dry white-cell pipette. The latter is then filled with the agglutinating serum. After the pipette is shaken vigorously for a few seconds, the contents are transferred to a serological tube and centrifuged in an electric table centrifuge at 1500 r.p.m., the machine being turned off automatically at the end of 2 minutes.

The sedimented red cells are dispersed by flicking the tube sharply several times by hand and shaking for an additional 45 minutes on an electric shaker[1] at 176 vibrations per minute. (The table of the shaker in our laboratory is covered with a 2-inch slab of fiberglass to insulate the specimens from the heat of the motor.)

Finally, the tube is flicked sharply to suspend the cell sediment, the larger clumps are permitted to settle for a couple of seconds, and a sample of the supernatant fluid is transferred to the hemocytometer by means of a white-cell pipette filled to the 1.0 mark. Counts are made in duplicate and usually agree within 10 per cent.

The free-cell counts are calculated to the number per cubic millimeter of uncitrated blood. With experimental mixtures of O and A erythrocytes or OM and ON in the proportion of 1:30, the lesser component can be determined by selective agglutination within 5 per cent.

4. *Transfusion procedure*

In the majority of our red-cell survival experiments since 1944, the practice has been to determine the blood volume of the recipient prior to transfusion and to determine accurately the quantity of blood administered by

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[1] No. 3623 rotating apparatus, obtainable from A. H. Thomas Company, Philadelphia.

weighing the container before and after the transfusion. The hemoglobin, hematocrit and total red-cell count also are determined on a sample taken from the bottle immediately before the experiment. The number of donor cells per mm.[3] of the recipient's blood immediately after transfusion, therefore, can be quite accurately calculated.

If the number of cells found is considerably less than the theoretical number, as is often the case when preserved blood is administered, the discrepancy is assumed to represent the number of the donor's erythrocytes eliminated by the recipient during the transfusion.

The volume of citrated blood administered in our experiments formerly was 450 ml., but since 1944 has usually been about 200 ml.

RESULTS

1. *Influence of agitation on the rate of agglutination*

The rate of combination of antigen with antibody is known to be extremely rapid. Hence, as numerous workers have shown, the accelerating effect of various modes of agitation and sedimentation on the rate of agglutination is due chiefly to increasing the incidence of contact between the sensitized cells. The size and shape of the container also may influence the rate especially if shaking is the mode of agitation.

Figure 1 illustrates the influence of the rate of horizontal shaking on the completeness of agglutination of erythrocytes at the end of 90 minutes with our apparatus. The unagglutinated-cell count is expressed as the number of free cells observed in the entire field of the hemocytometer, *i.e.*, in 0.9 mm.[3] of cell suspension. The effect of agitation above 190 vibrations per minute could not be determined with our facilities, but presumably a maximum eventually would be reached beyond which agglutination would be less complete and the damaging effect of the shaking would become a serious factor. Experiments with higher, but unknown, rates of shaking invariably have revealed considerable hemolysis. The results of shaking also vary somewhat with the blood, the antiserum, and the mode of agitation.

Agglutination may be accelerated greatly by an initial light packing of the cells by centrifugation. Figure 2 gives a comparison of the degrees of agglutination with and without centrifuging and at 2 rates of shaking. It will be observed from curve D that centrifugation, followed by shaking for 30 minutes, produces as complete agglutination as does shaking alone at 176 vibrations per minute at the end of 6 hours. Attempts to reduce the unagglutinated-cell count by agitation for more than 6 hours invariably resulted in visible hemolysis. Centrifuging also tends to damage red cells, and, if too prolonged or too rapid, will result in early hemolysis on subsequent shaking.

2. *Influence of strength of antiserum*

In determining the agglutinating titre of a strong antiserum by the ordinary method of progressive dilution, one tube in the series usually shows more rapid agglutination than those preceding or following it. This particular dilution represents, or approximates, the concentration of optimum proportion of the antigen and antibody as far as rate of agglutination is concerned. If aggregation of the

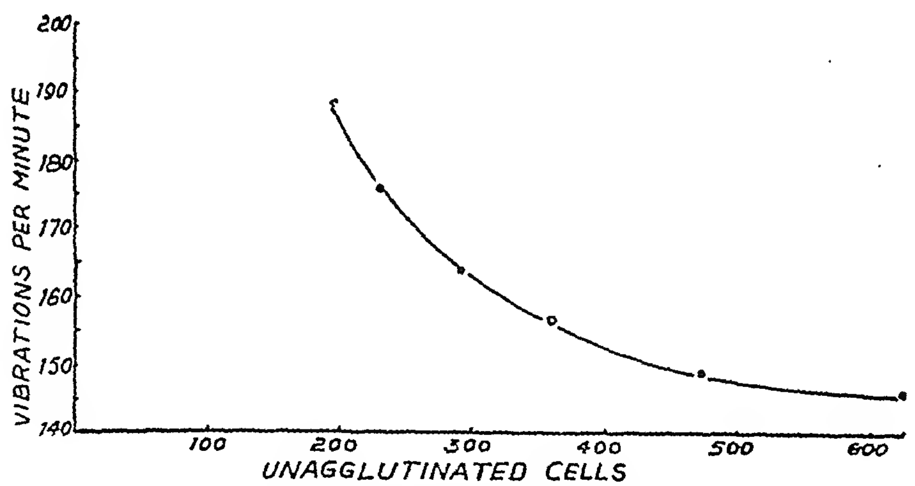

FIG. 1. INFLUENCE OF THE RATE OF SHAKING ON THE COMPLETENESS OF AGGLUTINATION

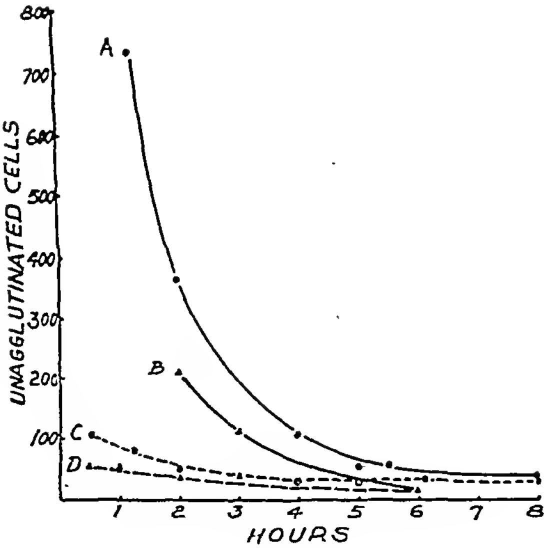

FIG. 2. PROGRESS OF AGGLUTINATION AT TWO RATES OF SHAKING WITH AND WITHOUT AN INITIAL LIGHT CENTRIFUGATION

Curves A and C, 146 vibrations per minute; curves B and D, 176 per minute.

red cells takes the form of a single, compact clump, one easily may be misled into assuming that agglutination is complete. On examination under the microscope, however, a surprisingly large number of unagglutinated cells may be observed. The concentration which affords the most rapid agglutination, therefore, may not produce the most complete reaction. This is true especially of antisera of marked avidity, notably anti-M and anti-N sera, which, even when diluted many times, may produce visible and compact agglutination within a few seconds. In preparing an antiserum for selective agglutination, therefore, it is essential to ascertain the relative concentration which produces the most complete aggregation of cells. This concentration usually is in the neighborhood of that which produces most rapid agglutination. With serum of only moderate potency, the maximum is obtained with the undiluted material.

The relation between the strength of the antiserum and the completeness of agglutination or the free-cell count is illustrated in Figures 3 to 9 inclusive. To facilitate comparison, the data are presented in the form of graphs. In doing so the authors wish to direct attention to the inconsistency in the results with different dilutions of serum rather than to imply that the fluctuations are due to variation in the properties of the erythrocytes or the antiserum, although either, or both the latter conceivably may occur.

(*a*) CONSTANCY OF THE UNAGGLUTINABLE-CELL COUNT

Figures 3 to 6 inclusive illustrate the fluctuations in the free-cell count with various dilutions of antiserum and in different individuals, over a period of 2 months. The results are expressed as thousands of cells per cubic millimeter of the recipient's blood. Attention is directed to the magnitude of the fluctuations in Figure 6. The counts were done in duplicate.

The variability of the free-cell count with the lower concentrations of antiserum is pronounced. With serum of marked avidity, *e.g.*, anti-M and anti-N, the rapidity, firmness, and compactness of agglutination may easily mislead one into thinking that agglutination is complete.

The stronger concentrations, on the other hand, produce a low free-cell count, which, in the case of the optimum concentration, remains practically constant within the counting error. Occasionally we have found that the recipient's free-cell count, after the donor's cells have disappeared from the circulation, is slightly higher than before the transfusion. In these cases the higher count has been used in calculating the results. For the purpose of the red-cell survival test, provided a strong serum is used, the unagglutinable-cell count of the recipient may be assumed to remain constant.

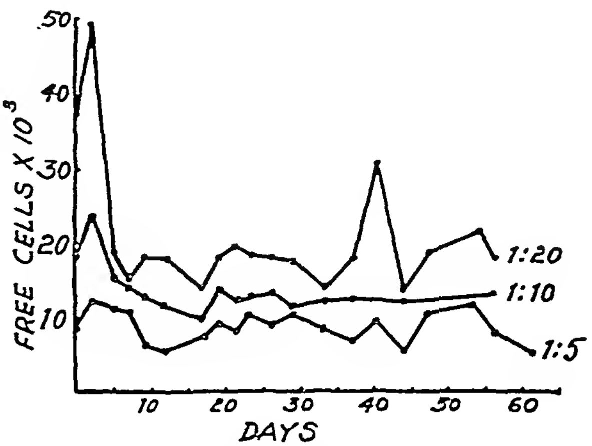

FIG. 3. VARIATION IN THE FREE-CELL COUNT OF A GROUP-B SUBJECT WITH POTENT ANTI-B SERUM (TITRE 5,000) DILUTED 5, 10, AND 20 TIMES, RESPECTIVELY

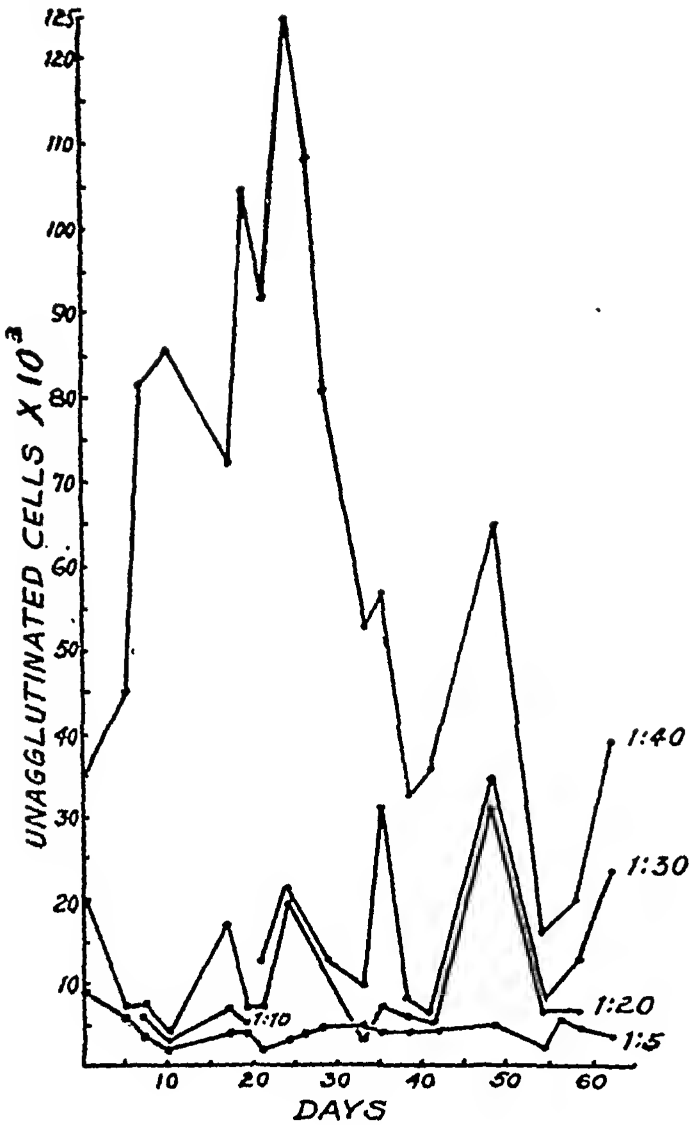

FIG. 4. VARIATION IN THE FREE-CELL COUNT OF A GROUP-A SUBJECT WITH A POTENT ANTI-A SERUM (TITRE 5,000) DILUTED 5, 10, 20, 30, AND 40 TIMES, RESPECTIVELY

(b) AGGLUTINABILITY OF PRESERVED ERYTHROCYTES

It was anticipated that if the fluctuations observed in the agglutinability of erythrocytes with weak sera are due to changes in the agglutinogens on the cell membrane, the agglutinability of the cells might become increasingly erratic with storage.

In Figure 7, the curves "A" represent periodic free-cell counts on blood (group $A_1MRh_1Rh_2$) preserved in citrate-dextrose solution at 5° C. over a period of 50 days; "B", the counts on fresh blood from the same donor. Here again the stronger serum (anti-A diluted 1:5) gave more constant results than the weaker lot (diluted 1:10). It is noteworthy also that under optimum conditions of preservation the agglutinability of the stored erythrocytes does not differ from that of the fresh blood. The counts are expressed as the number of free cells in the hemocytometer field in order to accentuate the fluctuations. (The factor for converting the count to cells per mm.[3] is approximately 270.) Since many of the erythrocytes, by the fiftieth day of storage, become nonfunctional (at least 50 per cent of the cells), it appears that agglutinability is not greatly impaired by the loss of viability provided conditions of preservation are favorable.

The curve in Figure 8 illustrates the behavior of the free-cell count in a sample of preserved blood of group OMRh using a potent anti-M serum diluted 20 times. The variation is typical of that observed with diluted antisera even in fresh blood. There is no evidence in this and in similar experiments that the reactivity of the M and N agglu-

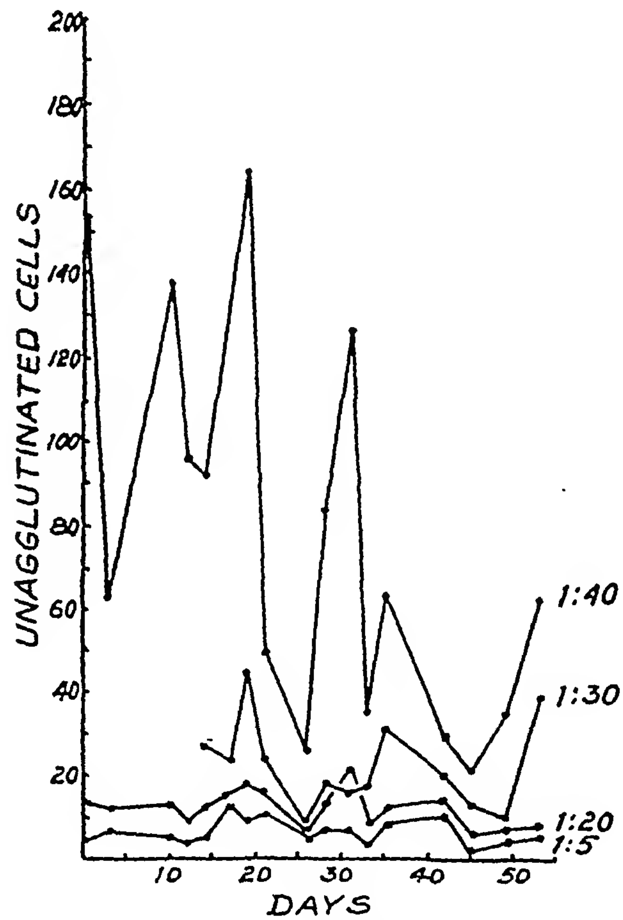

FIG. 5. FLUCTUATION IN FREE-CELL COUNT OF A GROUP-A INDIVIDUAL WITH POTENT ANTI-A SERUM (TITRE 5,000) DILUTED 5, 20, 30, AND 40 TIMES, RESPECTIVELY

tinogens is impaired during storage under optimum conditions at least up to 6 weeks.

3. *Estimation of transfused erythrocytes*

In view of certain anomalies observed up to 1944 in our experiments on mental patients it was thought advisable to perform a few cell-survival tests on normal subjects. Accordingly 200 ml. of fresh citrated blood of a donor group ONRh$_1$ was administered to each of 2 recipients and counts were followed daily for about 60 days. Figure 9 illustrates the disappearance of the donor's cells from the circulation of a recipient, group OMNRh$_2$, as determined with anti-M serum (titre 64), and Figure 10, the curve for the second recipient, group AMRh$_1$Rh$_2$, as determined simultaneously with anti-A (dotted curve) and anti-M sera. The gaps in the curves were occasioned by a holiday. It is noteworthy that despite the ir-

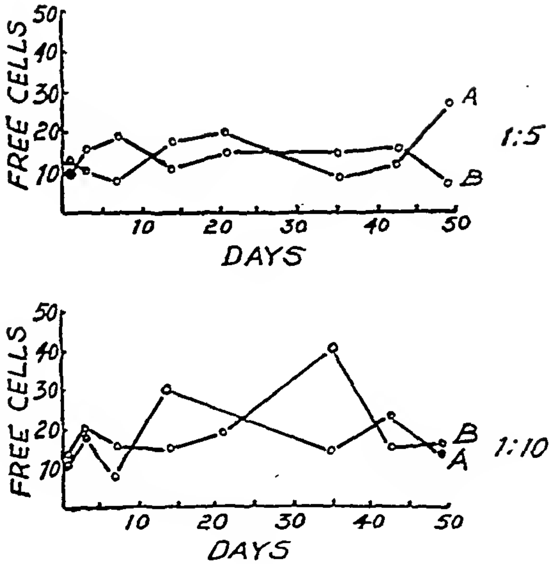

FIG. 7. COMPARATIVE VARIATION IN THE FREE-CELL COUNT IN PRESERVED AND FRESH BLOOD OF A GROUP-A SUBJECT USING POTENT ANTI-A SERUM (TITRE 5,000) DILUTED 5 AND 10 TIMES, RESPECTIVELY

Curves A represent preserved blood and curves B, fresh blood.

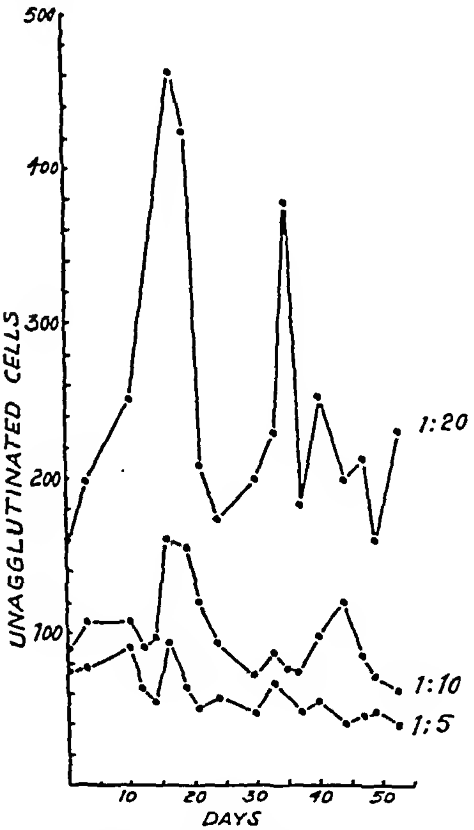

FIG. 6. FLUCTUATION IN FREE-CELL COUNT OF A GROUP-A INDIVIDUAL WITH A POTENT ANTI-A SERUM (TITRE 5,000) DILUTED 5, 10, AND 20 TIMES, RESPECTIVELY

regularities due to the use of diluted sera, the course of the red-cell elimination is quite accurately indicated. With diluted antisera, cell counts should be performed at least weekly in order to be able to correct for irregularities.

Figure 11 represents a transfusion series, performed in June 1944, in which 150 ml. of blood from 1 donor of group ON, was administered to each of 4 recipients. The first received a fresh specimen and the others, in turn, samples that had been preserved in citrate-dextrose at 5° C. for 12, 22, and 40 days, respectively. The free-cell counts of the recipients are included in Table I.

The rapid elimination of non-viable red cells within 3 to 6 hours after transfusion is indicated in curves B, C, D. In our experience, with blood stored for more than 3 weeks, there usually is indication of a partial elimination of the non-viable cells even during the period of the transfusion (200 ml.). The 100 per cent count in the post-transfusion sample in recipient D, therefore, is exceptional. It is noteworthy that a rapid elimination of erythrocytes always occurs with preserved blood within a few hours after transfusion, the loss amounting roughly to 1 per cent of the cells per day of storage (or more if conditions of preserva-

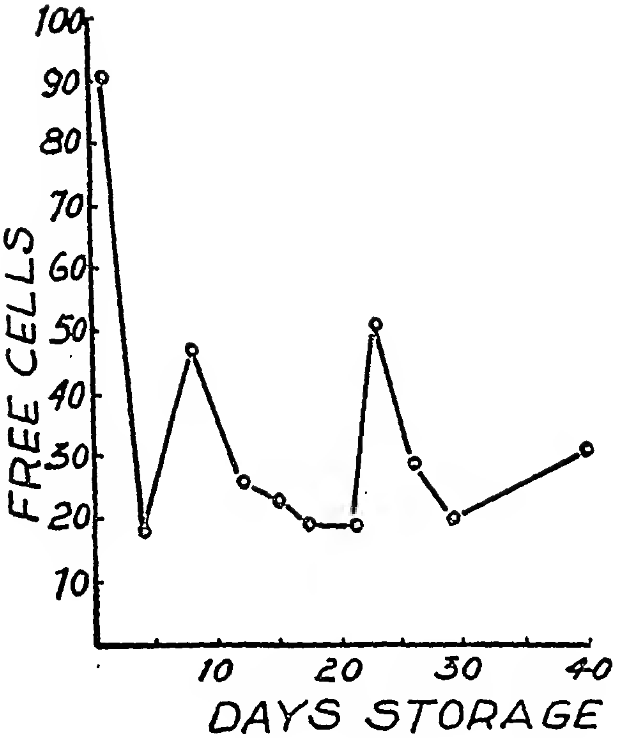

FIG. 8. FLUCTUATION IN THE FREE-CELL COUNT OF A PRESERVED BLOOD SAMPLE, GROUP-AM, USING AN AVID, BUT DILUTED, ANTI-M SERUM

tion are not optimum). With strong antisera the survival curves, for the most part, are free from gross irregularities.

One type of anomaly which the writers have encountered occasionally in exaggerated form with potent anti-M and anti-N, and even with

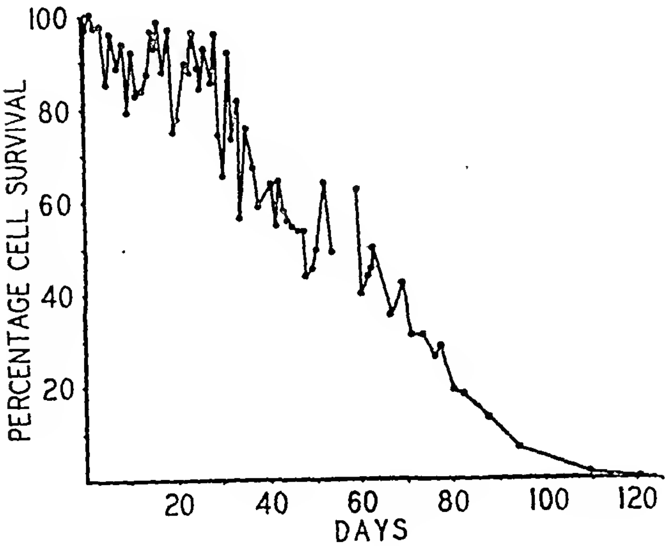

FIG. 9. ELIMINATION OF THE ERYTHROCYTES OF A GROUP-ON DONOR FROM THE CIRCULATION OF A GROUP-OMN RECIPIENT USING DILUTED ANTI-M AGGLUTINATING SERUM

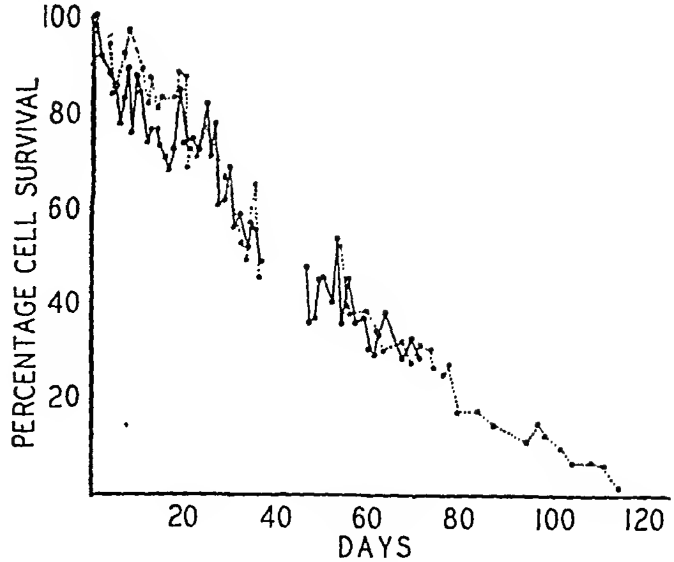

FIG. 10. ELIMINATION OF THE ERYTHROCYTES OF A GROUP-ON DONOR FROM THE CIRCULATION OF A GROUP-AM RECIPIENT, COUNTS BEING MADE SIMULTANEOUSLY WITH ANTI-A (DOTTED CURVE) AND ANTI-M SERA

strong anti-A serum, is illustrated by the large discontinuities in Figures 12, 13, and 14. The particulars of these experiments are given in Table II.

It will be observed that the free-cell counts of one of the recipients of type M differs considerably from the others. The same anti-M serum was used in each case. It is noteworthy also that the large deviation in Figure 13 was given by the recipient with the lower free-cell count. The irregularities in the 3 figures exhibit a remarkably smooth course, a circumstance which makes explanation difficult on the basis of technical errors.

DISCUSSION

Of the various factors that may influence the accuracy of selective agglutination, the strength of the antiserum is of major concern. As yet there is no practical method for characterizing a serum quantitatively as to its avidity and agglutinin content. The titre method of estimating potency gives only comparative values. Nevertheless, it is valuable for testing the strength of the serum for typ-

TABLE I

Recipient	Group	Antiserum	Free-cell count
			per mm.^{3}
A	OMN	anti-M	4,000
B	AMN	anti-M	4,000
C	OMN	anti-M	5,000
D	AMN	anti-M	7,000

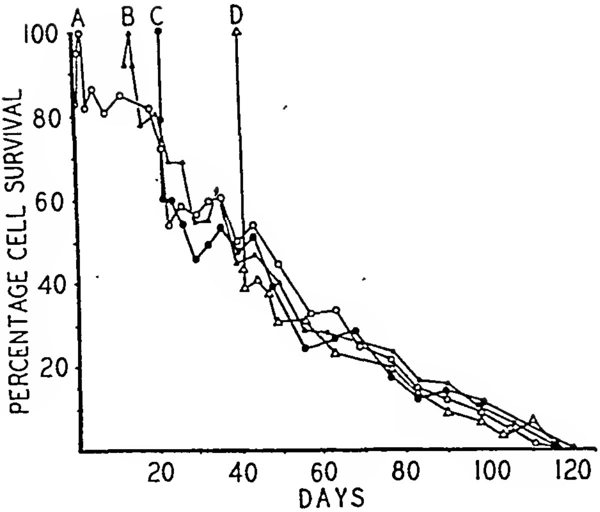

FIG. 11. ELIMINATION OF THE ERYTHROCYTES OF A DONOR, BLOOD GROUP-ON, FROM THE CIRCULATION OF 4 RECIPIENTS, USING A STRONG ANTI-M AGGLUTINATING SERUM. (SEE TABLE I.)

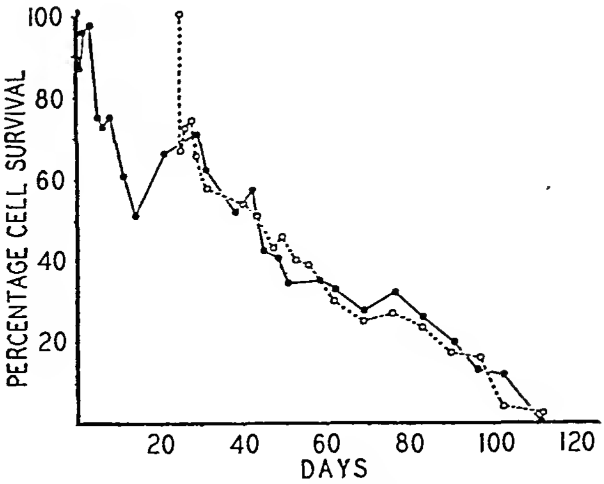

FIG. 12. ELIMINATION OF THE ERYTHROCYTES OF A FRESH AND A PRESERVED BLOOD SAMPLE FROM A GROUP-ON DONOR, FROM THE CIRCULATION OF 2 GROUP-OM RECIPIENTS. (SEE TABLE II.)

ing purposes and for estimating the optimum concentration of red cells and antiserum.

The term "optimum," as applied to agglutination, usually implies rapidity of reaction rather than completeness. The point of optimum proportion in the titre series, for example, is the concentration of cells and serum which produces the most rapid or most compact agglutination. As mentioned previously, this concentration does not necessarily produce maximum agglutination and, with sera of marked avidity, *e.g.*, anti-M and anti-N, the number of unagglutinable cells may be relatively large. Rapidity, compactness and firmness of agglutination, therefore, are not reliable criteria of the completeness of the reaction.

As shown in the foregoing graphs (Figures 3 to 9) antisera, particularly diluted sera, which give a high free-cell count, usually display marked variability. It is noteworthy, however, that the free-cell count even with undiluted sera may vary with different bloods. Thus, in Table II, the free-cell count with one individual was 5,000 and with another, was 18,000. Occasionaly also, one meets with a potent serum which gives a constant free-cell count with one person and a variable count with another. There are instances when dilution of the serum reduces the free-cell count, *e.g.*, when the serum concentration is above the optimum. In general, however, one can say that a high concentration of agglutinin is necessary for maximum and consistent agglutination, and that dilution of the serum tends to increase the number of unagglutinated cells and the variation in the counts.

TABLE II

Figure	Donor	Recipient	Storage period	Anti-serum	Recipient's free-cell count
			days		*per mm.*^{3}
12	ON	$OMRh_1$	fresh	anti-M	4,000
		$OMRh_2$	25		3,000
13	Pooled $ONRh^+$	$OMRh_1Rh_2$	22	anti-M	18,000
	$ONRh_1$	$OMRh_1$	22		5,000
14	$OMRh_1$	$ONRh_2$	21	anti-N	42,300

The reason for the high free-cell count with markedly avid antisera is not clear. Anti-M and anti-N sera, like many heterologous immune sera, produce a very rapid and firm type of agglutination. It is significant that the unagglutinated cells apparently do not adhere to the agglutinates already formed nor are they occluded to any considerable extent among the rapidly aggregating masses. The failure of many of the cells to become agglutinated cannot be attributed to a lack of antibody. Centrifugation will cause further aggregation but not to the extent one would expect with fully sensitized cells.

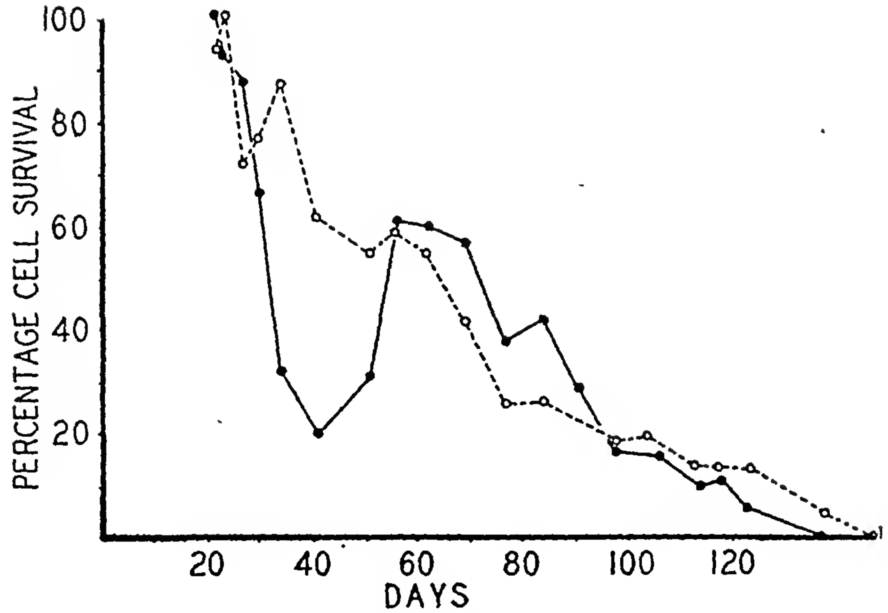

FIG. 13. ELIMINATION OF PRESERVED ERYTHROCYTES OF 2 GROUP-ON DONORS (POOLED SAMPLE) FROM THE CIRCULATION OF 2 GROUP-OM RECIPIENTS. (SEE TABLE II.)

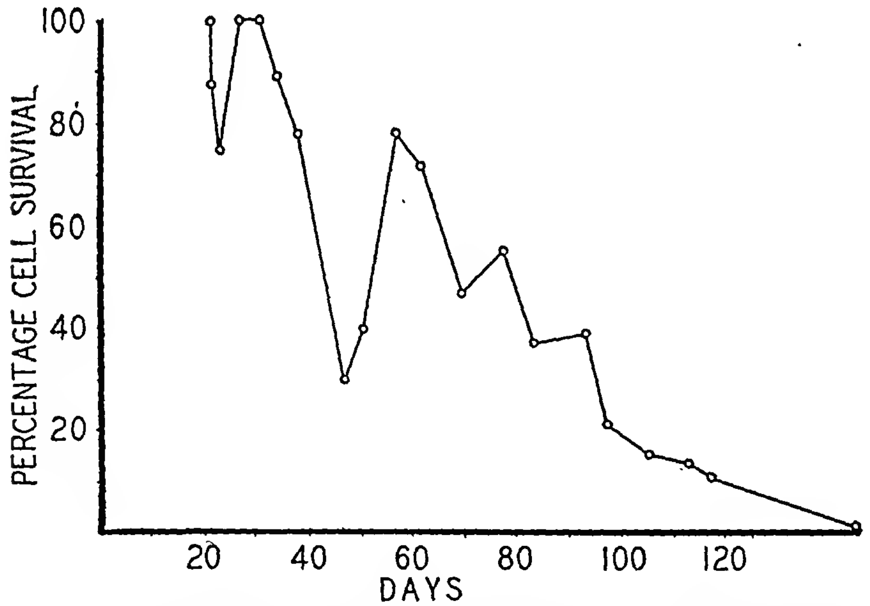

FIG. 14. ELIMINATION OF PRESERVED ERYTHROCYTES OF A GROUP-OM DONOR FROM THE CIRCULATION OF A GROUP-ON RECIPIENT. (SEE TABLE II.)

On first thought, the failure of some cells to be agglutinated with strong antiserum suggests that the red-cell population possibly may be made up of cells ranging in agglutinability from extremely to feebly reactive, and that, while the latter may be loosely agglutinated by centrifuging, like weak Rh agglutinates, they may be dispersed again by agitation. Such a hypothesis would imply that the so-called "unagglutinable" cells may be deficient in the content, distribution, or quality of their agglutinogens due perhaps to imperfect formation.

In opposition to this reasoning, one may point out that the addition of a strong antiserum to the residual free cells after agglutination with a weak antiserum, does not effect as complete agglutination as when the strong serum is applied directly to fresh cells. Weak serum may give rise to agglutinates in the form of doublets, or chains which can be broken by shaking. Addition of stronger antiserum does not consolidate the aggregates into

a compact mass. It is significant also that 2 volumes of half-strength antiserum are not necessarily equivalent in agglutinating strength and effect to 1 volume of undiluted serum for a given volume of cell suspension even when centrifugation is employed. On the basis of these observations, one is inclined to attribute the differences in free-cell count and agglutinability to a reversible change in the state of the agglutinins or other components of the system with dilution and changes in temperature.

The variability in the counts with diluted antiserum is of considerable interest since the fluctuations are not due to systematic errors. In the writers' experience the free-cell counts are likely to vary from day to day but they may be quite consistent on any day.

While the use of diluted, though avid, anti-M and anti-N sera explains some of the sharp irregularities observed in our earlier study, the larger and more regular deviations of the type illustrated in Figures 12, 13, and 14 are not so easily explained. Our former interpretation, that perhaps some of the donor's erythrocytes are sequestered from the recipient's circulation and released later, has been abandoned. The deviations doubtless are connected in some way with reversible changes in the properties of the antiserum, or possibly to reversible changes in the reactivity of the recipient's cells. It is evident from Table II that they are not attributable to the use of weak antiserum, nor are they confined to the use of the M and N blood types. Other factors such as agitation, temperature, salt concentration, and pH have been ruled out. The study of this phenomenon is being continued.

Highly potent anti-A and anti-B sera being easy to prepare, or being readily obtainable the writers recommend for selective agglutination, sera which give a free-cell count not exceeding 10,000 per $mm.^3$ The lowest free counts obtained in our studies were about 2,500. With strong serum the variation in the free-cell count is usually within 5,000 per $mm.^3$ Irregularities in the cell-survival curve become noticeable when the free-count exceeds 15,000, and, above 40,000, fluctuations may occur ranging up to 200,000 from day to day. Obviously it is desirable to keep the variation within the limits of error of the technique. With small transfusions, *e.g.*, 200 ml., it is especially important to select antisera which will reduce the free-count to a minimum.

CONCLUSIONS

From the evidence submitted and additional work not reported herein, it appears that there is no routine method for achieving complete agglutination of erythrocytes and that such an attainment is not possible when agitation is involved. In some experiments, however, the unagglutinable-cell count has been reduced to as low as 2,500 per $mm.^3$ With strong agglutinating serum the free-cell count of the individual remains practically constant for the majority of persons, especially of group A_1. The sharp irregularities observed in the cell-survival curves in an earlier study are attributable to the use of diluted anti-M and anti-N sera. The larger and more regular type of deviation, however, still remains unexplained; it is doubtless connected with some obscure change in the properties of the anti-serum or of the recipient's red cells. These deviations and irregularities do not necessarily vitiate the results of the cell-survival method. A high degree of accuracy can be obtained in the estimation of survival if the blood volume of the recipient and the number of erythrocytes transfused are accurately known.

SUMMARY

The presentation concerns the factors that affect the accuracy of the selective-agglutination procedure for estimating the survival of erythrocytes after transfusion. The influence of agitation, centrifugation, and the agglutinin titre of the antiserum on the rate and completeness of agglutination of red cells is discussed. The unagglutinable-cell count cannot be eliminated, but it can be reduced to a minimum and practically constant quantity by careful selection of the antiserum. Some of the irregularities observed in an earlier study undoubtedly were due to the use of diluted, though avid, anti-M and anti-N sera. The larger and more regular deviations, however, are not so easily explained; they appear to be related to reversible changes in the properties of the antiserum or to reversible changes in the properties of the recipient's red cells. If the blood volume of the recipient and the number of red cells transfused

are accurately known, the progressive elimination of the donor's cells from the circulation of the recipient can be followed with a fairly high degree of accuracy.

ACKNOWLEDGMENTS

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The authors are indebted to Dr. L. K. Diamond for a supply of high-titred anti-A and anti-B serum, and to Dr. A. S. Weiner for anti-M and anti-N sera, and for Rh-typing.

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BIBLIOGRAPHY

1. Ashby, W., The determination of the length of life of transfused blood corpuscles in man. J. Exper. Med., 1919, 29, 267.
2. Schaefer, G., and Wiener, A. S., Limitations in use of preserved blood for transfusions; preliminary report. Quart. Bull. Sea View Hosp., 1939, 5, 17.
3. Wiener, A. S., and Schaefer, G., Limitations in use of preserved blood for transfusion; study of fate of transfused erythrocytes in recipient's circulation. M. Clin. North America, 1940, 24, 705.
4. Mollison, P. L., The survival of transfused erythrocytes in hemolytic disease of the newborn. Arch. Dis. Childh., 1943, 18, 161.
5. Wiener, A. S., and Peters, H. R., Hemolytic reactions following transfusions of blood of the homologous group, with 3 cases in which the same agglutinogen was responsible. Ann. Int. Med., 1940, 13, 2306.
6. Mollison, P. L., and Young, I. M., Survival of transfused erythrocytes of stored blood. Lancet, 1940, 2, 420.
7. Bushby, S. R. M., Kekwick, A., Marriott, H. L., and Whitby, L. E. H., Survival of stored red cells after transfusion. Lancet, 1940, 2, 414.
8. Belk, W. P., and Barnes, B. C., Survival time after transfusion of erythrocytes of citrated human blood stored at 4° to 6° centigrade. Am. J. M. Sc., 1941, 201, 838.
9. Denstedt, O. F., Osborne, D. E., Stansfield, H., and Rochlin, I., Survival of preserved erythrocytes after transfusion. Canad. M. A. J., 1943, 48, 477.
10. Wearn, J. T., Warren, S., and Ames, O., Length of life of transfused erythrocytes. Arch. Int. Med., 1922, 29, 527.
11. Maizels, M., and Paterson, J. H., Survival of stored blood after transfusion. Lancet, 1940, 2, 417.
12. Loutit, J. F., Factors influencing preservation of stored red cells. J. Path. & Bact., 1945, 57, 325.
13. Thalhimer, W., and Taylor, E. S., Transfusion of centrifuged human type O cells resuspended and stored in 10 per cent corn syrup. J. A. M. A., 1945, 127, 1096.
14. Dacie, J. V., and Mollison, P. L., Survival of normal erythrocytes after transfusion to patients with familial haemolytic anaemia (acholuric jaundice). Lancet, 1943, 1, 550.
15. Mollison, P. L., and Young, I. M., On survival of transfused erythrocytes of stored blood. Quart. J. Exper. Physiol., 1941, 30, 313.
16. Wiener, A. S., Blood Groups and Transfusion. Third Edition. Charles C. Thomas, Springfield, Illinois, 1943.
17. Davidsohn, I., and Rosenfeld, I., Preparation of anti-M and anti-N testing fluids. Am. J. Clin. Path., 1939, 9, 397.
18. Landsteiner, K., The Specificity of Serological Reactions. Harvard University Press, Cambridge, Mass., 1945.
19. Boyd, W. C., Fundamentals of Immunology. Interscience Publishers, Inc., N. Y., 1943.
20. Eagle, H., Specific agglutination and precipitation; mechanism of reactions. J. Immunol., 1930, 18, 393.
21. Eagle, H., Specific agglutination and precipitation; velocity of reactions. J. Immunol., 1932, 23, 153.
22. Duncan, J. T., Specific character of stage of aggregation in agglutination and precipitation of antibody-antigen compounds. Brit. J. Exper. Path., 1938, 19, 328.
23. Kendall, F. E., Quantitative relationship between antigen and antibody in precipitin reaction. Ann. New York Acad. Sc., 1942, 43, 85.
24. Jones, F. S., and Orcutt, M., Prozone phenomenon in specific bacterial agglutination. J. Immunol., 1934, 27, 215.

THE PRESERVATION OF WHOLE BLOOD[1]

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The purpose of this paper[2] is to present the results of experimental work on the preservation of whole blood *in vivo* and *in vitro* carried out over a period of several years. Certain standards of preservation *in vitro* (the osmotic resistance) and *in vivo* (the serum bilirubin variations) have been carried out extensively in the early as well as in the late experiments, thus affording a means of comparison.

More specifically, it will present results obtained from comparative studies of several preserving solutions employing the radioactive iron technic.

The conclusions from early experimental work (1) can be summarized as follows:

(*1*) The absence of spontaneous hemolysis is not a satisfactory proof of good preservation of erythrocytes of stored blood.

(*2*) The fragility of erythrocytes to hypotonic salt solution is a dependable measure of blood preservation and parallels the *in vivo* studies provided that the preserving solution does not contain dextrose in high concentration.

(*3*) Serum bilirubin determinations before and after transfusion are readily available and constitute a reliable index of the survival of transfused erythrocytes.

(*4*) The determination of the total bile pigment is a good means of measuring post-transfusion destruction of erythrocytes.

(*5*) The safe period of preservation at 4° C. of blood collected in plain sodium citrate solution is not over 5 days.

(*6*) Experimental work has shown that the addition of glucose to sodium citrate solution does increase the period of safe preservation of whole blood (2 to 6) but generally not enough to compensate for the disadvantage of the greater dilution of the plasma proteins.

(*7*) The administration of blood collected in a solution with a high volume of water and relatively high glucose content (4, 6) has been found to be followed often by an increase in serum bilirubin even when the blood is fresh. This bilirubinemia does not increase noticeably for the first few days of storage, suggesting that it probably has to do with the immediate effect of glucose on a certain type of cells, the least resistant, possibly either the youngest or the oldest.

In the present work the radioactive iron technic was applied to the study of whole blood preservation along with other means.

RADIOACTIVE IRON TECHNIC

The iron isotope 55, with a half life of 5 years, was used in these experiments. This material was synthesized into ferric ammonium citrate in neutral aqueous solutions. Two donors were selected for preparation with radioactive iron. They were both healthy young white males belonging to the "O" blood group, Rh positive and with low anti-A and anti-B titre. Each received 12 intravenous injections of 10 ml. of radioactive ferric ammonium citrate at 2- to 3-day intervals over a 4-week period. The preparation of the iron isotopes used in this work and the radioactivity of blood samples were carried out by Gibson *et al*, as a cooperative plan outlined elsewhere (7). At the end of this period, one donor (SD–1) had a unit activity of 15.35 and the second (SD–2), a unit activity of 15.8.

The interpretation of the results is based on the assumption that as long as the cell membrane remains intact there is no interchange of radioactive iron between the cells and plasma, therefore the cells become "tagged" for the duration of their life.

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[1] This paper is the 19th of a series on blood, plasma, and plasma substitutes from the Laboratory of Clinical Pathology of the Bryn Mawr Hospital. The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Bryn Mawr Hospital, and with the aid of the Bryn Mawr Research Foundation.

[2] To avoid useless repetitions, references to previous work and to work published in this series of papers have been kept to a minimum.

At the end of the 12 intravenous injections, the radioactivity titre of the donor blood was high enough to permit bleeding and transfusing into recipients.

Technic of bleeding, division into aliquots, and storage

The donors were bled of 400 to 500 ml. into the solution to be tested. This was precooled and by mechanical means maintained in constant gentle motion during collection and for 3 minutes after completion of bleeding. The blood immediately after mixing was divided by a closed system into a number of aliquots, of approximately equal size, and these were stored at 4° C. except for 1 specimen, which was used for immediate transfusion.

Administration of blood

The aliquots from each donor were administered to different recipients. The recipients used were all hospital patients known to have a normal erythropoiesis. All were convalescing and ranged in age between 18 and 86.

Proper grouping, Rh typing, and cross matching were carried out in all instances. Blood volume measurements, hemoglobin, and hematocrit determinations were also carried out. All recipients were Rh positive.

The transfused blood was well mixed by gentle rotation before and during the administration. The rate of transfusion was maintained at about 5 ml. per minute, the whole procedure requiring 24 to 30 minutes. The amount of blood given to the various recipients was measured volumetrically by calibration of each bottle before and after transfusion. All the blood remaining in the tubing and filter was washed into the recipient by saline solution immediately following the blood transfusion. The amount of blood remaining in the bottle, usually amounting to 10 ml. was carefully measured after removal with measured amounts of saline solution and used for radioactivity measurements. The exact number of packed red cells given was also determined by this method.

Samples of blood were taken from each of the recipients within 1 minute of the completion of the transfusion, ½ hour and 3 hours after transfusion, and twice daily for 5 days and at the end of the seventh day. These samples were withdrawn with new syringes and needles and without stasis. The blood was drawn into a double oxalated tube, and the hematocrit was determined. The red cells were then "laked" with 30 ml. of distilled water and used for radioactivity measurements. The patient's blood volume was measured with the dye method (8 to 11) and also calculated from the unit activity of the donor's blood and the unit activity of the first sample after transfusion.

The cell survival was calculated from the unit activity of the donor's blood, the volume of cells given, the unit activity of the recipient's blood, and the volume of the recipient's blood cells.

Citrate-glucose solution (McGill No. 1)

The 2 radioactive donors were bled of about 400 ml. into 200 ml. of McGill solution No. 1. This latter solution was prepared by using 80 ml. of a 3.2 per cent solution of sodium citrate and 120 ml. of a 5.4 per cent dextrose solution. These 2 solutions were sterilized separately and mixed in the proper proportion before use in the standard donor bottles.

The results are expressed as per cent of cell survival against time (see Figure 1). The survival of erythrocytes using the fresh blood appeared to be optimal in recipient SR–1. In recipient SR–2, however, a drop of 10 per cent of the transfused blood apparently occurred in the first 24 hours with further cell loss in the first 7 days to a total of about 16 per cent. Erythrocytes from blood stored at plus 4° C. for 10 days appear to be definitely less resistant. An average of 50 per cent of the cells appear to be lost from the circulation within the first 24 hours. This confirms findings obtained by the determination of the serum bilirubin and total bile pigment. The state of preservation of erythrocytes from blood stored for 20 and 30 days appears to be extremely poor. Eighty per cent or more of the erythrocytes from 4 samples appears to be destroyed within the first 3 hours.

No untoward reactions were encountered in any of the transfusions given.

A.C.D. solution (Rapoport)

The formula suggested by Rapoport is as follows:

Sodium citrate ($2H_2O$) .	1.66 grams
Citric acid (H_2O)	0.59 gram
Distilled water	100.00 ml.

To 90 ml. of this solution 22.5 ml. of 15 per cent dextrose were added in the collection bottles and the mixture was sterilized by autoclaving for 20 minutes at 120° C.

Four hundred and fifty ml. of the donor's blood were collected in the above A.C.D. solution according to the method described above. The blood was then divided by a closed system into 4 aliquots of approximately 150 ml. each.

The 4 aliquots from each donor were transfused into 4 human recipients at the following intervals of time: immediately, 10 days, 20 days, and 30 days in one case; and immediately, 10 days, 21 days, and 31 days in the other.

The results of this experiment are summarized in Figure 2.

With fresh blood, in the first 24 hours after transfusion, recipient SR–9 showed a drop of 10 per cent of the transfused blood and recipient SR–13 showed a drop of 6 per cent. After 10 days of storage, recipient SR–10 showed a 10 per cent drop in 24 hours and SR–14, a 9 per cent drop. With blood stored 20 and 21 days respectively, SR–11 showed a drop of 61 per cent in the first 24 hours and SR–15, a drop of 58 per cent. At the end of 30 and 31 days of storage, in recipient SR–12 there was a drop of 89 per cent in the transfused cells and in recipient SR–16, a drop of 85 per cent in the first 24 hours following transfusion.

The preservation of erythrocytes with this A.C.D. solution is good for a period of storage up to 10 or 11 days. The erythrocyte survival after 20 to 21 days' storage is very poor.

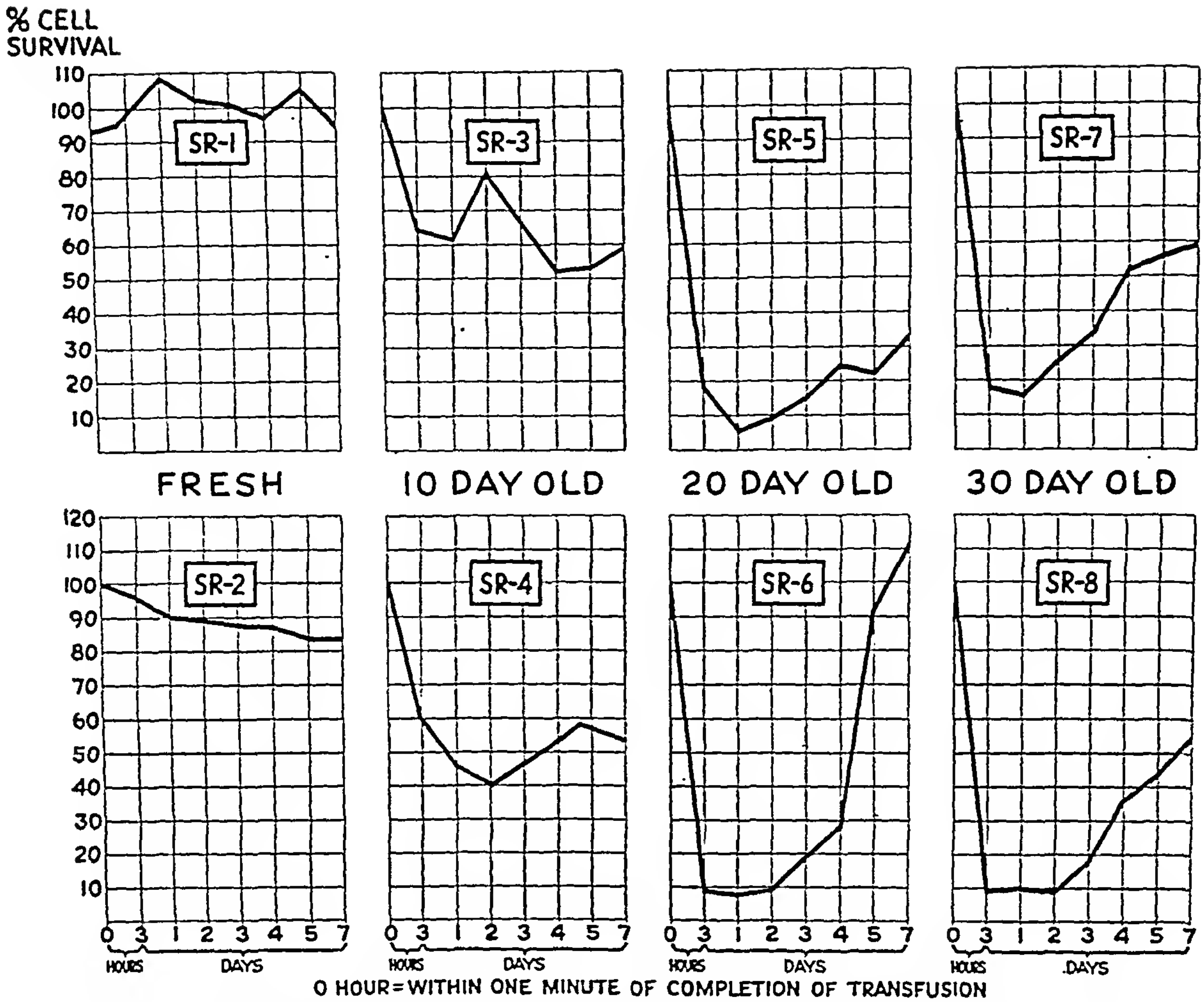

FIG. 1. POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES DETERMINED BY RADIOACTIVE IRON. WHOLE BLOOD PRESERVED IN CITRATE-GLUCOSE SOL. WITHOUT BUFFER (MCGILL SOLUTION NO. 1)

Citrate-glucose solution (Baxter-Alsever)

The citrate-glucose solution (Baxter-Alsever) was furnished by the Baxter Company ready for use. The formula is as follows:

Trisodium citrate	0.8 per cent
Dextrose	2.05 per cent
Sodium chloride	0.42 per cent
Water	500 ml.

pH 6.0

Four hundred and forty-three ml. of the donor's blood were placed in 500 ml. of this solution. The blood was then stored at plus 4° C. for 13 days. At the expiration of this period of time the blood was thoroughly mixed and divided into 2 aliquots of approximately equal size. The blood was then administered immediately to 2 recipients. After 13 days of preservation, the survival of the erythrocytes transfused was 70 per cent and 64 per cent respectively. In both recipients hyperbilirubinemia occurred.

Recipient SR–17 was a male, aged 54, convalescing from a subtotal gastrectomy, weight 60 kgm., height 170 cm. This patient received 132.8 ml. of packed erythrocytes and the survival measured with the radioactive iron technic showed within 3 hours of transfusion a drop of 21 per cent, indicating the destruction of 27.8 ml. of packed erythrocytes. The serum bilirubin before transfusion was 0.7 mgm. per cent, 3 hours after transfusion it rose to 1.0 mgm. per cent, and after 7 hours to 1.8 mgm. per cent; 24 hours after transfusion it had returned to normal, 0.7 mgm. per cent (see Figure 3).

Recipient SR–18 was a female, aged 47, convalescing from skin grafting, weight 71.8 kgm., height 162.5 cm. This patient received 107 ml. of packed erythrocytes and the survival measured with the radioactive iron technic showed a loss of 19 per cent of the transfused erythrocytes at the end of 3 hours, indicating a loss of 20.3 ml. of packed red cells. The patient's serum bilirubin before transfusion was 0.5 mgm. per cent; 3 hours after, it was 1.3 mgm. per cent; 7 hours after, it was 0.7 mgm. per

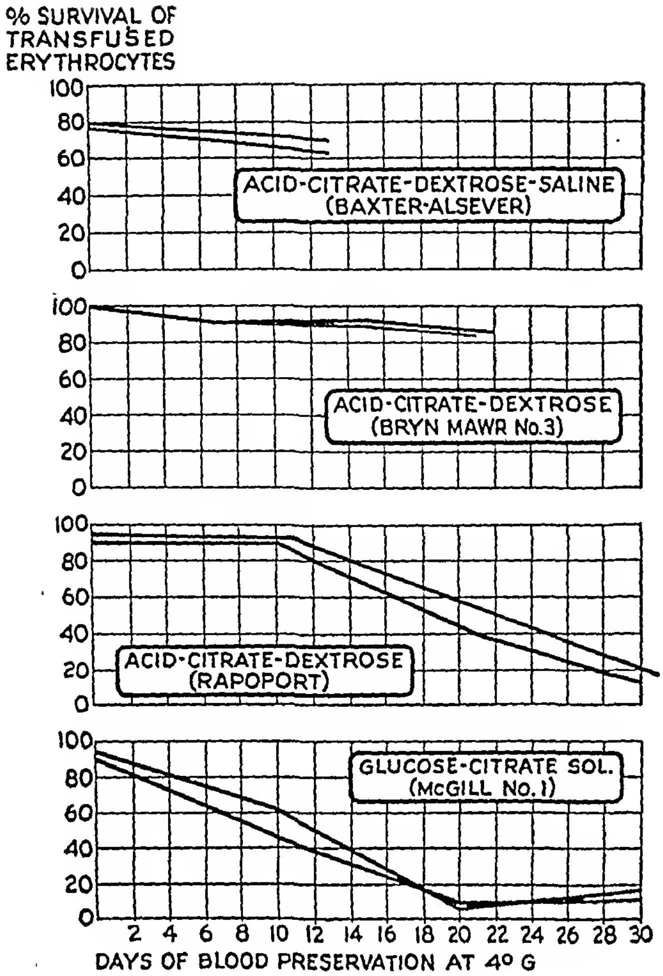

FIG. 2

cent; and 24 hours after, it was 0.8 mgm. per cent. As control, a third recipient SR-20 received a comparable amount of blood preserved in B.M. A.C.D. No. 3 for the same period of time. The radioactive iron study showed a good preservation with a destruction within 3 hours of transfusion of less than 5 ml. of packed erythrocytes. There was no significant change in this patient's serum bilirubin level.

It can be said in conclusion that with the Baxter-Alsever solution optimal preservation of whole blood is less than 13 days, probably not more than 1 week.

A.C.D. solution (*Bryn Mawr No. 3*)

The formula is as follows:

Sodium citrate (trisodium dihydric)	2.1 grams
Citric acid (monohydric)	0.66 gram
Dextrose (anhydrous)	2.0 grams
Water	100.0 ml.

75 ml. of this mixture contains:

Sodium citrate	1.6 grams
Citric acid	0.50 gram
Dextrose	1.5 grams

Five hundred ml. of blood from the same 2 donors were collected in 75 ml. of this solution according to the above technic. Each sample was divided into 4 equal aliquots by a closed system and stored at plus 4° C. until ready for use.

The 4 aliquots from each donor were transfused according to the technic described into 4 human recipients at varying intervals of time: immediately, 7 to 8 days, 14 to 15 days, and 21 to 22 days.

The recipients selected for use were similar to those used above, and their ages varied from 37 to 79.

The results are summarized in Figure 2.

With the injection of fresh blood there appears to be no immediate loss of cells. After 7 to 8 days, the loss is 10 per cent; after 14 to 15 days of preservation, the loss remains at 10 per cent; and after 21 to 22 days, it is only 16 per cent.

DISCUSSION

The radioactive iron technic as applied to the study of whole blood preservation has given results comparable to those previously obtained from the study of the serum bilirubin level before and after transfusion and from the measure of the total bile pigment output.

The data show that with the addition of dextrose to sodium citrate and with variable amounts of water to obtain a final dextrose concentration in the plasma of 1.45 per cent (McGill No. 1) to 1.28 (Baxter-Alsever), the period of preservation is increased over that obtained with plain citrate solution. The increased period of preservation is in any case relatively small. If the standard of optimal preservation of 90 per cent and 70 per

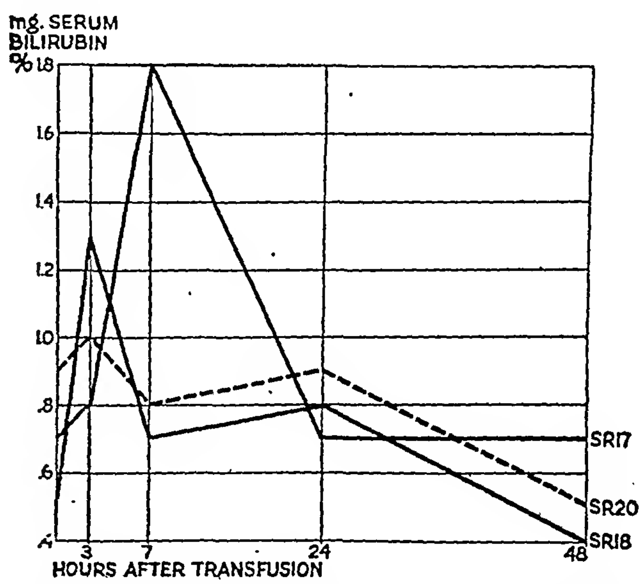

FIG. 3. HYPERBILIRUBINEMIA OCCURRING IN PATIENTS RECEIVING BLOOD PRESERVED FOR THIRTEEN DAYS IN A CITRATE-GLUCOSE SOLUTION (BAXTER-ALSEVER)

cent minimal is adopted, McGill solution No. 1 is good only for about 5 days and the Baxter-Alsever for approximately 12 to 14 days.

The acid citrate-dextrose solutions tested gave a far better preservation. The solution with less glucose (.4 per cent Bryn Mawr No. 3) gave a distinctly better preservation than the solution with more glucose (.88 per cent Rapoport).

In comparing the maximal length of satisfactory erythrocyte preservation with various solutions obtained by the different observers using a comparable technic, it will be noted that the results obtained by our group at Bryn Mawr Hospital are generally indicative of a shorter survival of the erythrocytes after transfusion. This is most likely due to the fact that at the Bryn Mawr Hospital all recipients were convalescent patients, whereas in most other studies the recipients were normal adult males. The implication of a more rapid erythrocyte destruction is borne out by other observations, particularly the higher total bile pigment output in diseases.

In addition to the means of study mentioned, we have repeatedly attempted to follow the fate of transfused red cells by means of the Ashby technic (12) as modified by Denstedt (13). The results which we have obtained are very difficult to interpret. The major difficulty, encountered by other experimenters, is due to the fact that the count of non-agglutinable cells varies a great deal. We have noted that non-agglutinable cells seem to disappear from the circulation suddenly and for irregular periods of time; their reappearance is usually at an unpredictable level. Under these circumstances, to draw a line and to interpret the slope of such line as meaning progressive loss of the transfused cells appears to us to be somewhat arbitrary. We have not been able to correlate the findings obtained with the technic of Dr. Denstedt with the results obtained with the studies of the bilirubin level following transfusion and with the results obtained from the study of the total bile pigment output in the days immediately following the transfusion nor with the results obtained with the radioactive iron technic. We are, therefore, not reporting in this paper the results of the studies on cell preservation using the non-agglutinable cell technic.

CONCLUSIONS

A.C.D. solutions are a better blood preservative than citrate or glucose-citrate solutions. With the best formulas optimal preservation can be secured for at least 22 days.

BIBLIOGRAPHY

1. Strumia, M. M., The fate of transfused refrigerated blood and the problem of blood banks. S. Clin. North America, 1942, 22, 1693.
2. Rous, P., and Turner, J. R., The preservation of living red blood cells *in vitro*. I. Methods of preservation. J. Exper. Med., 1916, 23, 219.
3. Perry, M. C., Preservation of blood for transfusion. Wisconsin M. J., 1926, 25, 123.
4. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. I. Various factors influencing hemolysis. J. A. M. A., 1940, 114, 850.
5. Maizels, M., and Whittaker, N., Effects of storage on human erythrocytes. Lancet, 1940, 1, 113.
6. Alsever, J. B., and Ainslie, R. B., New Method for preparation of dilute blood plasma and operation of complete transfusion service. New York State J. Med., 1941, 41, 126.
7. Gibson, J. G., 2nd, Aub, J. C., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., and Sack, T., The measurement of post-transfusion survival of preserved stored human erythrocytes by means of two isotopes of radioactive iron. J. Clin. Invest., 1947, 26, 704.
8. Gibson, J. G., 2nd, and Evans, W. A., Jr., Clinical studies of the blood volume. I. Clinical application of a method employing the azo dye "Evans blue" and the spectrophotometer. J. Clin. Invest., 1937, 16, 301.
9. Gibson, J. G., 2nd, and Evelyn, K. A., Clinical studies of the blood volume. IV. Adaptation of the method to the photoelectric microcolorimeter. J. Clin. Invest., 1938, 17, 153.
10. Gibson, J. G., 2nd, and Evans, W. A., Jr. Clinical studies of the blood volume. II. The relation of plasma and total blood volume to venous pressure, blood velocity rate, physical measurements, age and sex in ninety normal humans. J. Clin. Invest., 1937, 16, 317.
11. Price, P. B., and Longmire, W. P., Use of T-1824 in plasma volume determinations. Bull. Johns Hopkins Hosp., 1942, 71, 51.
12. Ashby W., The determination of the length of life of transfused blood corpuscles in man. J. Exper. Med., 1919, 29, 267.
13. Denstedt, O. F., Osborne, D. E., Stansfield, H., and Rochlin, I., Survival of preserved red cells after transfusion. Anesthesiology, 1944, 5, 237.

THE PRESERVATION OF ERYTHROCYTES LEFT OVER FROM PLASMA PREPARATION[1]

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The practice of transfusion of red cells left over from preparation of plasma has gained some popularity (1 to 11). Whether or not this practice will ever become an accepted and widespread procedure and what its practical merits are need not be discussed at this time.

It is obvious that the use of red cells left over from plasma preparation is possible only when plasma is separated after a relatively short period of time. It has been the practice to resuspend the red cells in crystalloid solutions of which the two most popular have been physiological salt solution (5) and 10 per cent corn syrup (12). Since the red cells normally are immersed in a colloidal protein fluid, it is natural to expect *a priori* a better preservation of the erythrocytes when maintained in a protein colloidal medium. This has been found to be true in the experimental work reported here. Ross (13) has shown good preservation of red cells left over from plasma preparation when the cells are simply stored in the remaining plasma. In this paper it will be shown that globin considerably improves the preservation of red cells and that its use as a resuspension medium for red cells is practical. This does not justify at present the conclusion that for optimal preservation of erythrocytes it is necessary to add any diluting fluid to the packed cells left over after the separation of plasma.

[1] This paper is the 20th of a series on blood, plasma, and plasma substitutes from the Laboratory of Clinical Pathology of the Bryn Mawr Hospital. The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Bryn Mawr Hospital, and with the aid of the Bryn Mawr Research Foundation.

PRESERVATION OF ERYTHROCYTES IN PHYSIOLOGICAL SALT SOLUTION

This resuspension medium has been the most commonly used. The maximum recommended period of preservation is from 5 to 6 days (10) at icebox temperature. We have found that the preservation of fresh red cells in physiological saline solution at 4° C. for more than a few hours very greatly reduces the post-transfusion survival of the red cells. Injection of 200 to 450 ml. of such cells in humans (corresponding to 500 to 1,000 ml. of whole blood) is followed in the majority of cases by hyperbilirubinemia and greatly increased bile pigment output.

In these experiments the blood was collected in plain sodium citrate solution (50 ml. of 4 per cent sodium citrate for each 500 ml. of blood), and immediately stored at 4° C. The plasma was separated after centrifugation and in any case within 36 hours from the time of collection. The red cell residue was immediately mixed with .9 per cent sodium chloride solution, chilled, in an amount approximately equivalent to that of the plasma removed. The red cell-saline mixture was immediately placed at 4° C. and was maintained there until time for use. Grouping, Rh typing and cross matching were carried out as usual. A typical case is reported here.

The patient was a man, aged 54, white. This patient was chosen because hematological studies, including a bone marrow biopsy and total bile pigment output determinations, had shown that the sole defect was a greatly reduced normoblastic activity of the bone marrow, from cause unknown. On first admission to the hospital on May 23, the hemoglobin concentration was 2.3 grams per cent with a reticulocyte count of .1 per cent and a total volume of circulating packed erythrocytes of only 280 ml. The patient belonged to the B group and was Rh negative. Sixteen whole blood transfusions of compatible blood, each containing on an average of 450 ml. of blood, were given to this patient over a 53-day period. The patient appeared to tolerate well the transfusions of whole citrated blood, showing no hyperbilirubinemia nor any other signs of reaction. At the time of his discharge on July 12 the hemoglobin concentration was 9 grams per cent. The

patient was readmitted to the hospital on July 25 and at this time the hemoglobin concentration was 6.8 grams per cent, with a reticulocyte count of .1 per cent. From blood volume measurements it is estimated that on July 12 the patient had a volume of packed erythrocytes of 986 ml. On July 29 the hemoglobin concentration was 6.1 grams per cent and the volume of erythrocytes was 745 ml. or a decline of 241 ml. of packed erythrocytes in 17 days with an average loss of about 14 ml. of packed erythrocytes daily. This loss is rather low and indicates good preservation of the transfused erythrocytes. Repeated serum bilirubin determinations showed a concentration of .8 mgm. per cent with a low urobilin output. This patient was given on July 29, 791 ml. of packed erythrocytes, of O group, Rh negative, separated from plasma within 18 hours of collection after centrifugation, mixed with chilled saline solution to make a total volume of 1,071 ml. and maintained at 4° C. for 72 hours.

Following the transfusion of the resuspended erythrocytes, the patient had a hemolytic reaction, with discomfort in the lower abdomen, passage of red urine, containing hemorrhagic casts, high urobilin concentration in the urine and a rise in the serum bilirubin to over 14 mgm. per cent. In 48 hours the patient was entirely well from the reaction (see Figure 1). On August 2, the hemoglobin concentration was 6.9 grams per cent and the volume of packed erythrocytes 758 ml., indicating practically a complete loss of the injected mass of erythrocytes. On August 2, the patient received 305 ml. of packed erythrocytes as 896 ml. of whole citrated blood

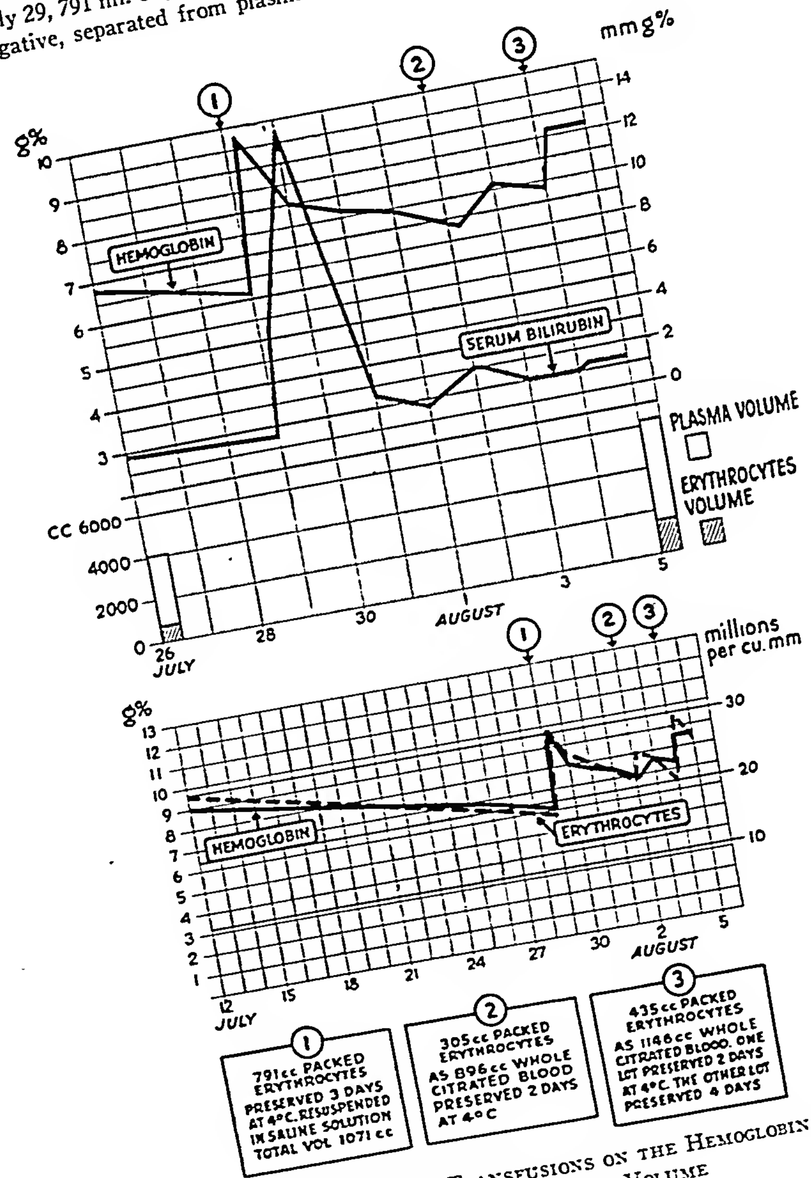

FIG. 1. THE EFFECT OF TRANSFUSIONS ON THE HEMOGLOBIN, SERUM BILIRUBIN AND BLOOD VOLUME

preserved for 2 days. On August 4, 435 ml. of packed erythrocytes as 1,146 ml. of whole citrated blood were given to the patient, one lot of blood being 2 days old and the other 4 days old. The patient had no apparent reaction to the transfusion. On August 5, the hemoglobin concentration was 8.6 grams per cent and the measured volume of packed erythrocytes was 1,538 ml. with an apparent gain of 780 ml. of packed erythrocytes. This represents for the period of time of observation a good survival of the injected red cells, the total amount of injected red cells having been 740 ml. As can be seen from Figure 1, the transfusion of whole citrated blood preserved from 1 to 4 days at 4° C. was not followed by a notable increase in the serum bilirubin level.

IN VITRO COMPARATIVE STUDIES ON THE PRESERVATION OF ERYTHROCYTES IN 4 PER CENT GLOBIN SOLUTION, SALT SOLUTION AND 10 PER CENT CORN SYRUP

The effect of a 4 per cent globin solution on the preservation of resuspended erythrocytes has been studied both *in vitro* and *in vivo*. The studies *in vitro* included pH determination, mean corpuscular volume, osmotic resistance, and the hemoglobin concentration in the supernatant fluid.

The addition of globin to a suspension of erythrocytes in salt solution does not appreciably alter the cell preservation. However, when the erythrocytes are suspended in a sodium citrate-dextrose solution, the globin exerts a definite beneficial effect on the preservation. We are well aware that the choice of crystalloids and their relative concentration as well as the final pH of the solution employed here are probably far from the ideal. We are, however, reporting a typical experiment to show the change which the addition of globin produces in the preservation of erythrocytes.

Five hundred ml. of blood from an adult healthy donor were collected in 60 ml. of a 4 per cent sodium citrate solution, chilled. The sodium citrate solution was chosen because it was the same used by the Red Cross bleeding centers. The blood was allowed to remain for 18 hours at 4° C. It was then centrifuged for 45 minutes at 1,800 times gravity. The plasma was carefully removed, leaving only about 20 ml. of it with the packed red cells. The red cell sediment was then carefully mixed with the remaining plasma, and by means of a sterile calibrated cylinder it was divided into aliquots. Each of these aliquots was mixed with an equal part of the various preserving fluids. The composition of these is given in the following table:

Salt solution:

Sodium chloride	.9 per cent

A.C.D. solution:

Sodium citrate (trisodium, dihydric)	1.66 grams
Citric acid (monohydric)	0.59 gram
Dextrose (anhydrous)	3.75 grams
Distilled water (pyrogen-free)	125 ml.

Corn syrup: 10 per cent sterile solution as obtained from Schering, Glatz Company.

Dextrose-citrate:

Dextrose (anhydrous)	2.4 grams
Sodium citrate (trisodium dihydric)	1.9 grams
Water	100 ml.

Globin-dextrose-citrate:

Globin	4 grams
Dextrose (anhydrous)	2.4 grams
Sodium citrate (trisodium, dihydric)	1.9 grams
Water	100 ml.

After mixing, the various red cell suspensions were cultured and divided into 10-ml. aliquots in hard glass, rubber-stoppered bottles. These were immediately placed at 4° C. and maintained at this temperature until the time of study.

The determination of the osmotic resistance was made by a modification of the technique suggested by Parpart (14); the mean corpuscular volume was done in duplicate; the pH was determined with a Beckman apparatus. The hemoglobin of the supernatant fluid was done after thorough mixing of the sample followed by centrifugation. All samples were cultured again at the end of the stated period of time.

ANALYSIS OF RESULTS

Mean corpuscular volume. There was swelling in all of the solutions used. This swelling was minimal with the citrate-dextrose solution and with the salt solution. It was maximal with the 10 per cent corn syrup (see Figure 2). By the fifth day of preservation and continuing on to the end of the experiment, there was a shrinkage of the cells to within the normal range, except for the A.C.D. solution in which there was an excessive shrinkage followed by a progressive swelling to normal size. The addition of globin to the dextrose-citrate solution appears to have a definite effect on the rate and range of volume changes.

Mean osmotic resistance of resuspended erythrocytes (fragility). Except for the sodium chloride, the mean osmotic resistance is considerably increased. This increase is minimal with the

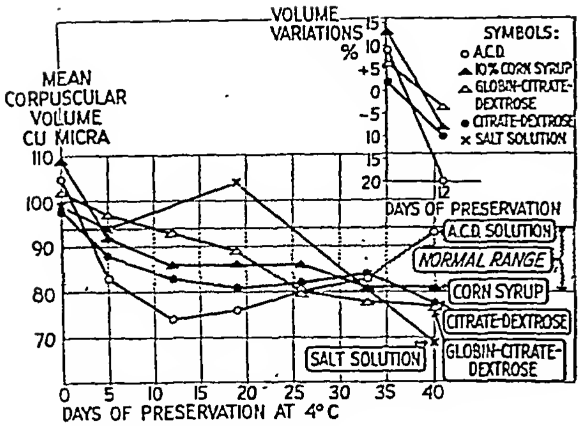

FIG. 2. MEAN CORPUSCULAR VOLUME

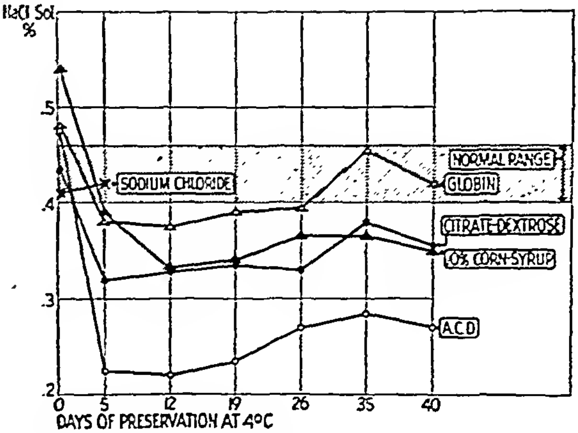

FIG. 3. MEAN OSMOTIC RESISTANCE OF RESUSPENDED ERYTHROCYTES (FRAGILITY)

globin citrate-dextrose solution, and it is maximal with the A.C.D. solution. The addition of globin to the citrate-dextrose solution definitely effects a change in osmotic resistance, maintaining it nearer to the normal change (see Figure 3).

pH variations. The variations of the pH of the original solutions of the supernatant fluid are indicated in the following table:

	A.C.D.	10 per cent corn syrup	Salt solution	Dextrose-citrate	Globin-dextrose-citrate
pH of original solution	5.04	4.22	5.52	6.05	7.71
pH of supernatant fluid					
0	6.0	7.3	7.4	7.1	7.2
5	6.0	7.5	7.7	7.05	7.4
12	6.0	7.6		7.05	7.2
19	6.0	7.6	7.4	7.0	7.2
26	6.0	7.6		7.0	7.25
33	6.0	7.5		7.0	7.2
40	6.0	7.4		7.0	7.2

Hemoglobin concentration in the supernatant fluid. To obtain this datum, the erythrocyte suspensions were thoroughly mixed and centrifuged; the determination was made by the micro method reported separately (15). The hemoglobin concentration in the A.C.D. solution, in the citrate-dextrose solution, and in the globin citrate-dextrose is comparable. It is definitely less in the 10 per cent corn syrup and increases much more rapidly in the salt solution. These data appear of little significance (see Figure 4).

POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES SUSPENDED IN 4 PER CENT GLOBIN SOLUTION BY THE RADIOACTIVE IRON TECHNIC

Clinical observations

Studies on the *in vivo* survival of erythrocytes resuspended in globin solution by means of the iron isotope technic have been carried out (see Figure 5).

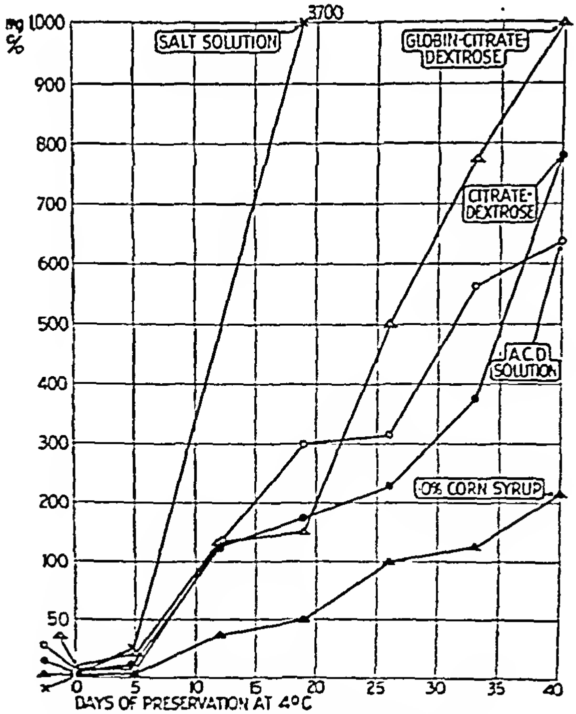

FIG. 4. HEMOGLOBIN CONCENTRATION IN THE SUPERNATANT FLUID

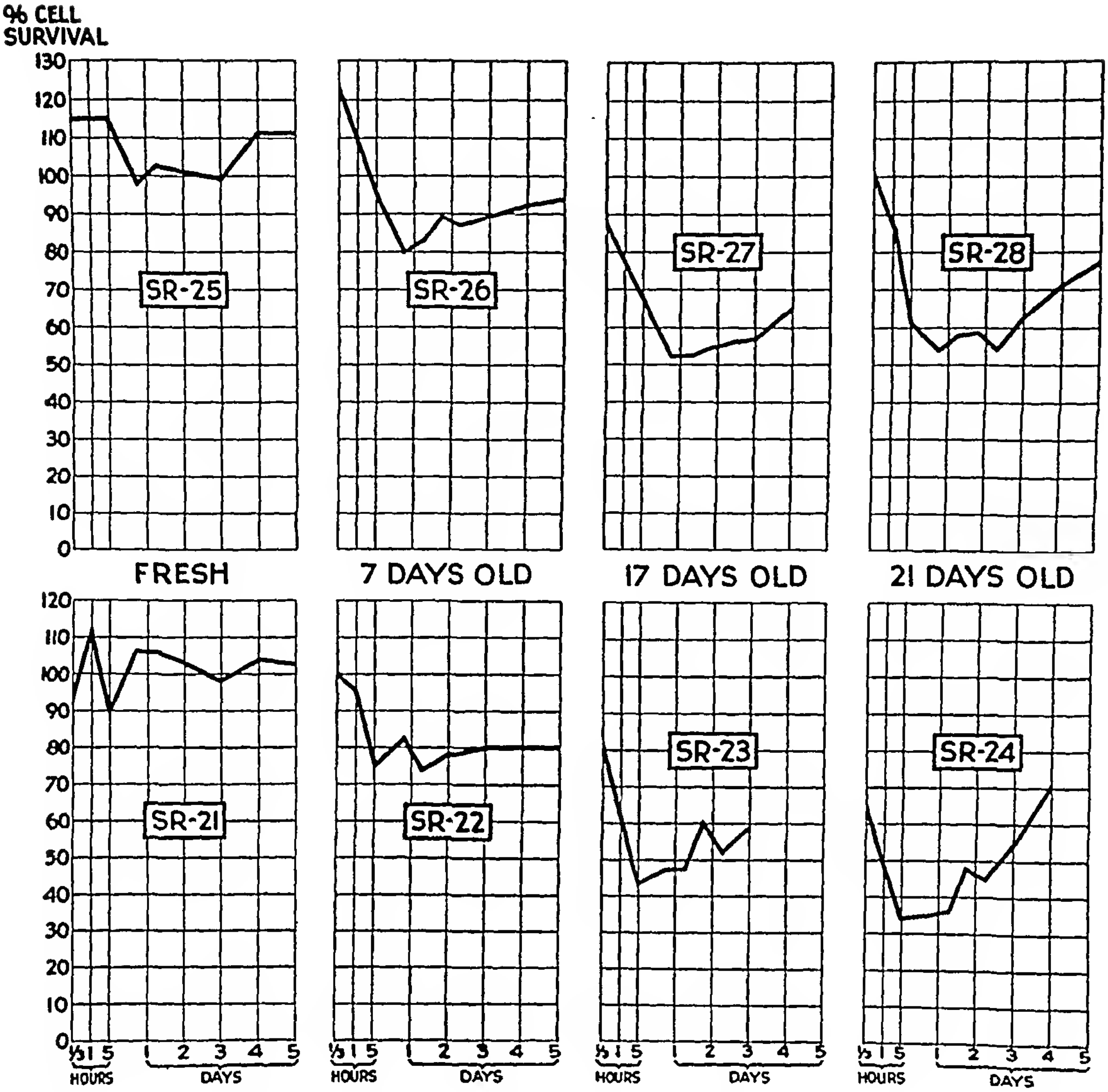

Fig. 5. Survival of Erythrocytes Resuspended in Citrate Dextrose-Globin Solution Determined by the Iron Isotope Technic

The survival of the transfused cells is 75 to 80 per cent up to 7 days of preservation and 50 per cent or better up to 15 days. It is of very great importance to note that:

(1) In our experience, using as recipients patients rather than healthy males as in the experiments reported by Gibson, the preservation of cells as determined by the iron isotope technic is generally poorer than that found by other observers, regardless of the preserving fluid employed.

(2) Whereas with all of the preserving fluids so far used the rate of cell deterioration is definitely accelerated, as the preservation is prolonged, with addition of globin it becomes definitely slower.

It is probable that an initial damage occurs and that a better choice of crystalloids to make the fluid iso-osmotic may very materially improve the preserving properties of the globin solution.

Figure 6 affords a comparison of the rate of preservation of erythrocytes resuspended in globin dextrose-citrate and of whole blood preserved in A.C.D. and glucose-citrate solutions.

The decline of the hemoglobin concentration noted in the days following was small and comparable to that noted previously. There appears to be no reason to encourage the use of erythrocytes preserved at 4° C. in saline solution for more than a few hours for the purpose of transfusion. As a matter of fact there appears to be no good reason for resuspending the red cell

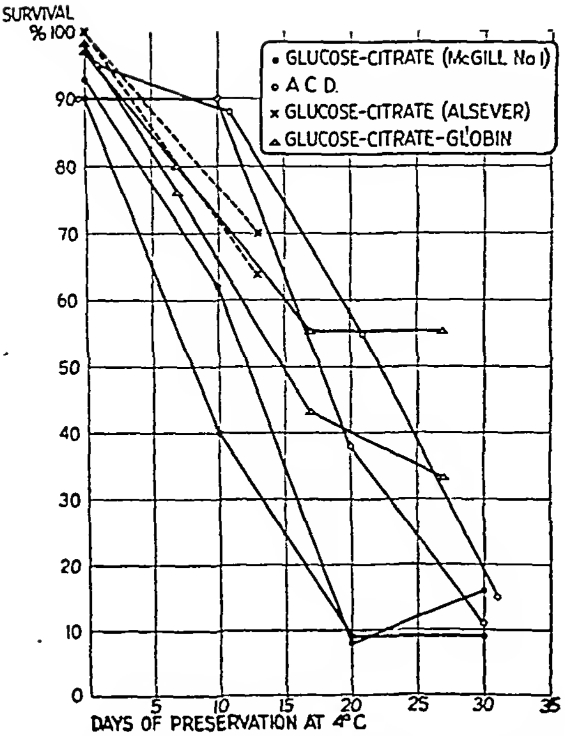

FIG. 6. SURVIVAL OF WHOLE BLOOD AND RESUSPENDED ERYTHROCYTES IN VARIOUS SOLUTIONS

residue in saline at all, as they can be transfused as they are, using an 18-gauge needle.

About 50 transfusions of 200 to 230 ml. of packed red cells, resuspended in globin solution, have been administered. Studies of the serum bilirubin content have been carried out. No hyperbilirubinemia has occurred, nor untoward reactions of any sort. The clinical results were those to be expected from well preserved cells.

CONCLUSIONS

The addition of globin in a 4 per cent concentration to an isotonic solution of dextrose-citrate increases the preservation of red cells left over from plasma preparation. With this mixture the preservation is optimal for 7 days, good up to 15 days.

BIBLIOGRAPHY

1. Strumia, M. M., Chornock, F. W., Blake, A. D., and Karr, W. G., The use of a "modified globin" from human erythrocytes as a plasma substitute. Preliminary report. Am. J. M. Sc., 1945, 209, 436.
2. Alt, H. L., Red cell transfusions in the treatment of anemia. A preliminary report. J. A. M. A., 1943, 122, 417.
3. Blum, L. L., The present-day status of combined blood-plasma bank, with reference to the use of concentrated red cell suspensions (operation and use in medium-sized hospital). J. Indiana M. A., 1943, 36, 187.
4. Blum, L. L., Red cell transfusions. J. A. M. A., 1943, 122, 892.
5. Cooksey, W. B., and Horwitz, W. H., Use of salvaged red cells. J. A. M. A., 1944, 124, 961.
6. Evans, R. S., The use of concentrated red cells as a substitute for whole blood in transfusion therapy of anemia. J. A. M. A., 1943, 122, 793.
7. Murray, C. K., Hale, D. E., and Shaar, C. M., The preparation and use of red blood cell suspensions in treatment of anemia. J. A. M. A., 1943, 122, 1065.
8. Semoff, M., Plasma and red blood cell banks for small institution. M. Rec., 1942, 155, 359.
9. Semoff, M., Red blood cells of plasma bank. Southwestern Med., 1943, 27, 248.
10. Taylor, E. S., Thalhimer, W., and Cooksey, W. B., The organization of a red blood cell transfusion service. J. A. M. A., 1944, 124, 958.
11. Watson, L., Red-cell suspension transfusions. Lancet, 1943, 1, 107.
12. Thalhimer, W., and Taylor, E. S., The transfusion of centrifuged human type O cells resuspended and stored in 10 per cent corn syrup. J. A. M. A., 1945, 127, 1096.
13. Ross, J. F., The *in vitro* preservation and post-transfusion survival of stored blood. J. Clin. Invest., 1947, 26, 687.
14. Parpart, A. K., Lorenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, A. M., The osmotic resistance (fragility) of human red cells. J. Clin. Invest., 1947, 26,
15. Karr, W. G., and Chornock, F. W., Technical note: Determination of hemoglobin in plasma (visual colorimetry). J. Clin. Invest., 1947, 26, 685.

AN ACID CITRATE–DEXTROSE SOLUTION WITH LOW WATER VOLUME AND LOW DEXTROSE CONCENTRATION [1]

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The blood preservative introduced by Loutit and Mollison (1) consisting essentially of a sodium citrate-dextrose mixture of relatively low pH, has proved superior to previously employed preserving fluids. The original formula and many subsequent modifications have a rather high volume. This produces considerable dilution of the plasma proteins.

The purpose of this study, begun in June 1944, was to obtain an A.C.D. solution of lower volume, so as to maintain an optimal concentration of plasma proteins, maintain or improve the capacity to preserve erythrocytes, and at the same time insure maximal stability of the resulting plasma.

The first solution tested was one proposed by Rapoport, as follows:

Sodium citrate (trisodium dihydric)	1.66 grams
Citric acid (monohydric)	0.59 gram
Distilled water	100 ml.

22.5 ml. of 15 per cent dextrose were added to 90 ml. of this solution, and the resulting solution was sterilized by autoclaving for 20 minutes at 120° C. This formula can be written as follows for the sake of comparison with other formulas:

A.C.D. Rapoport

Sodium citrate (trisodium dihydric)	1.50 grams
Citric acid (monohydric)	.55 gram
Dextrose	3.37 grams
Water	112.5 ml.

112.5 ml. of this solution were used for each 500 ml. of blood collected. The solution was cooled to 4° C. previous to use.

The post-transfusion survival of erythrocytes collected in this solution was tested with the radioactive iron technic (2). With fresh blood there was an immediate loss of 6 to 10 percent of the transfused cells. There was no increased loss of transfused cells after 10 days of preservation at 4° C. After 21 days of storage the survival dropped to 40 to 58 per cent and at the end of 31 days of storage the preservation was less than 20 per cent.

In a first modification of Rapoport's solution the amounts of citrate and glucose were slightly increased and the amount of water reduced. The formula was as follows:

Bryn Mawr A.C.D. No. 2

Sodium citrate (trisodium, dihydric)	1.6 grams
Citric acid (monohydric)	0.56 gram
Dextrose (anhydrous)	3.6 grams
Water to 100 ml.	

One hundred ml. of this solution were used for each 500 ml. of blood.

This solution was adopted for routine use in the hospital and by January 16, 1945, a total of 587 lots of blood had been collected in this solution. The results of 139 whole blood transfusions were thoroughly studied. It was concluded from these preliminary studies that this solution was no better than the previous one for preservation of erythrocytes, but it was found that the plasma obtained was somewhat more stable. The studies of the plasma composition will be reported later on. *In vitro* studies showed that the concentration of dextrose was much higher than the optimal. This conclusion was reached from studies of the osmotic resistance, mean corpuscular volume, and hemoglobin concentration in the supernatant plasma. Since this solution was not completely satisfactory, it was decided to study the effect of varying amounts of dextrose. At the

[1] This paper is the 21st of a series on blood, plasma, and plasma substitutes from the Laboratory of Clinical Pathology of the Bryn Mawr Hospital. The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Bryn Mawr Hospital, and with the aid of the Bryn Mawr Research Foundation.

same time the volume was reduced to 75 ml. Similar results were reported by Rapoport (3).

A basic formula was then adopted:

Sodium citrate (trisodium, dihydric)	1.6 grams
Citric acid (monohydric)	0.56 gram
Water	75 ml.

Varying amounts of dextrose were added to this basic formula as follows: 1.6, 1.8, 2.0, 2.2, 2.4 grams. Seventy-five ml. of the resulting solutions, chilled, were used for 500 ml. of blood.

IN VITRO STUDIES OF A.C.D. SOLUTIONS WITH VARYING AMOUNTS OF DEXTROSE

These included mean corpuscular volume, hemoglobin concentration in the supernatant plasma, and osmotic resistance. Samples of blood were prepared for *in vitro* studies as follows: 500 ml. of blood were obtained in a chilled donor bottle containing 1.6 grams of sodium citrate and .56 gram of citric acid in 50 ml. of water. Immediately after collection, the blood was divided into 5 equal aliquots and immediately transferred to 5 bottles, chilled, each containing 5 ml. of a solution of dextrose to yield the final concentration noted previously. A determination of the hemoglobin concentartion in the 5 samples was done to correct any inequality in the measuring of the samples. All figures given have been corrected. Samples of blood were taken from each solution to be tested immediately after mixing. The blood was then stored at 4° C. and similar samples were taken at the end of 7, 14, 21, and 28 days. Before sampling, the blood was thoroughly mixed by gentle rotation for a period of 5 minutes. At the completion of this time approximately 7 to 8 ml. of blood were removed aseptically and used for the various tests.

The technic used for the determination of the mean corpuscular volume is as follows: One ml. of blood was placed in a Wintrobe hematocrit tube and this was centrifuged at 3,600 r.p.m. for 30 minutes. The hematocrit was then read and the blood was again centrifuged at 3,600 r.p.m. for 10 minutes. If there was no change in the readings, the hematocrit was then read directly and an erythrocyte count was done on the same sample. The mean corpuscular volume was calculated in the usual manner.

The technic for the determination of the hemoglobin in the supernatant plasma is as follows: a 5-ml. sample of blood was centrifuged for 10 minutes at 1,800 r.p.m. At the end of this time the plasma was removed by the use of a capillary pipette and the hemoglobin determined as described elsewhere (4).

The technic used for determining the osmotic resistance is that suggested by Parpart (5), modified as follows: .05 ml. of whole blood was added to 5 ml. of the various solutions of Series I.[2] These mixtures were then placed in the water bath at 37° C. for ½ hour. At the end of this time the tubes were removed from the water bath and 5 ml. of each of the corresponding solutions of series II were added, the tubes were thoroughly mixed, centrifuged for 10 minutes at 3,600 r.p.m. The hemoglobin in the supernatant fluid was then estimated with the Klett photoelectric colorimeter. The hemoglobin content of the tube containing 0.1 per cent sodium chloride was considered as 100 per cent hemolyzed; the hemoglobin reading of the other tubes was recorded in per cent of this value. In Figure 2 sodium chloride concentration giving 50 per cent hemolysis are plotted against time of storage for the various preservative solutions.

Mean corpuscular volume

For the 5 solutions tested, those with the higher dextrose concentration showed an increasing fluctuation in the values of the mean corpuscular volume. The 2 highest concentrations of dextrose produced an abnormally high mean corpuscular volume of the stored cells. The pattern of variations for each concentration has been found to be fairly constant for blood from the same source (see Figure 1).

Osmotic resistance

For the first 3 weeks of preservation there was little difference between the cells preserved in the various concentrations of dextrose. A slight spread of values occurred after the twenty-first day of storage, the lower concentrations of glucose showing a better osmotic resistance than the higher (see Figure 2).

Hemoglobin concentration in supernatant plasma

There appears to be no difference up to the fourteenth day of storage between erythrocytes in the various concentrations of dextrose. A definite spread of values occurred at the end of the third

[2] Series I and Series II each consist of 10 tubes, each containing 5 ml. of a sodium chloride solution of the following concentration per cent:

Tube No.	Sodium chloride grams per cent Series I	Series II
1	0.100	1.900
2	0.300	1.700
3	0.400	1.600
4	0.450	1.550
5	0.500	1.500
6	0.550	1.450
7	0.600	1.400
8	0.700	1.300
9	0.900	1.100
10	1.000	1.000

Intermediate concentrations are used whenever necessary.

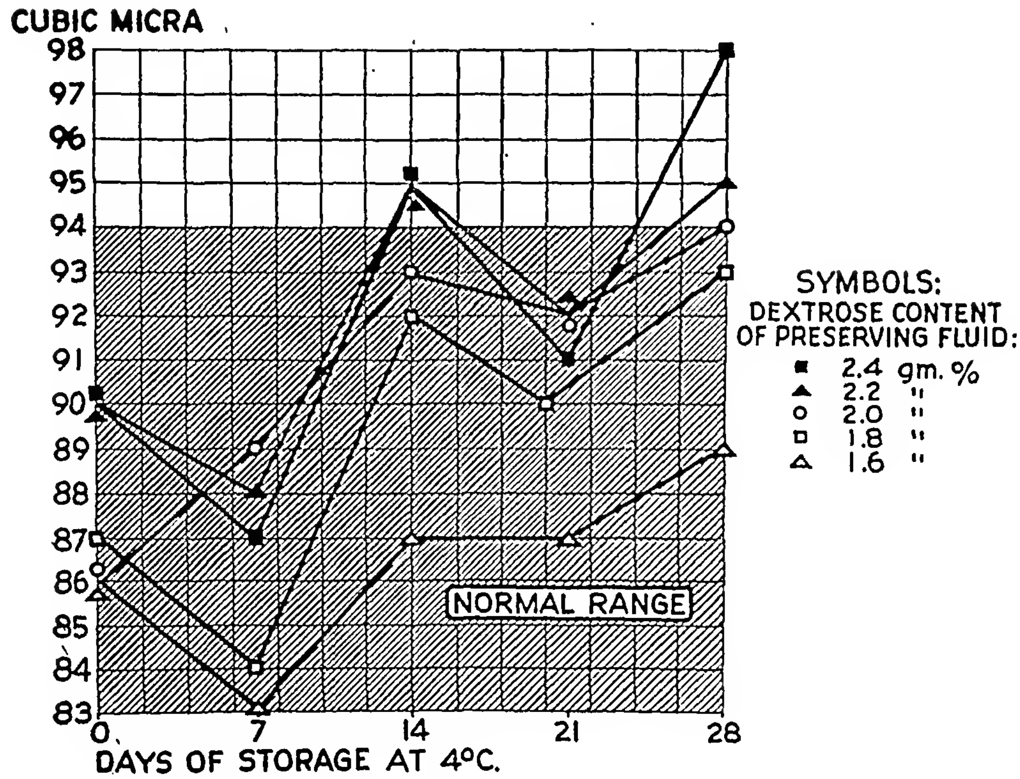

FIG. 1. EFFECT OF DEXTROSE CONCENTRATION ON THE MEAN CORPUSCULAR VOLUME

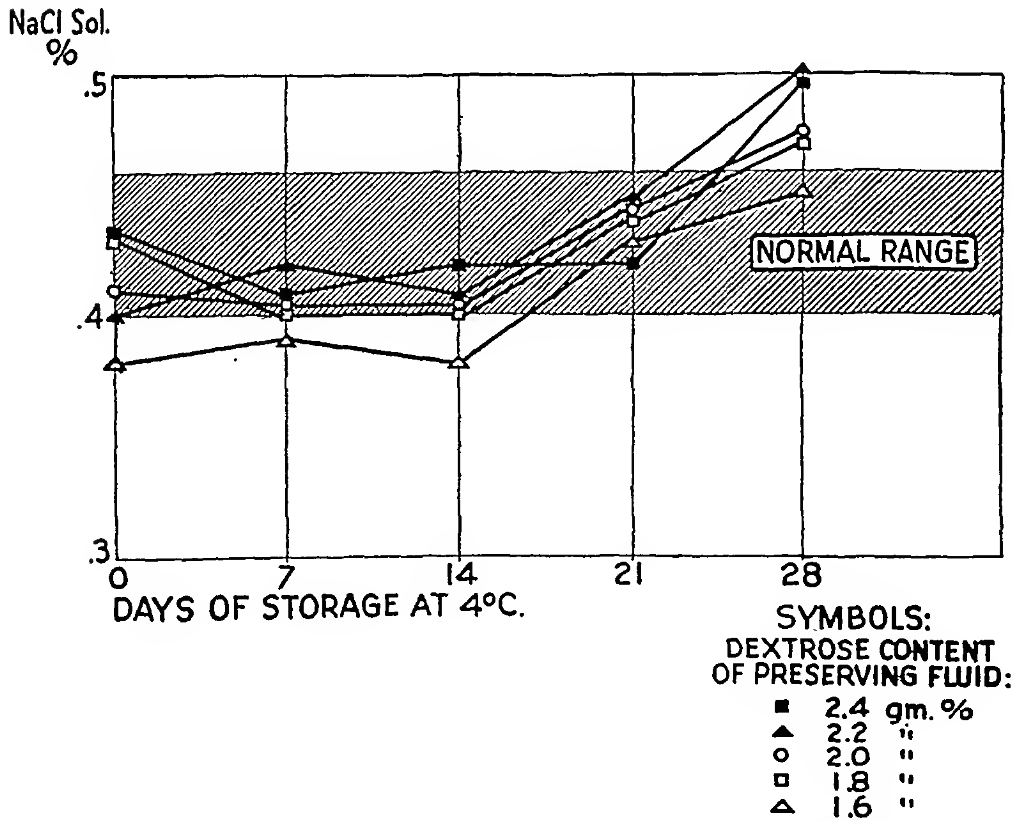

FIG. 2. EFFECT OF DEXTROSE CONCENTRATION ON THE OSMOTIC RESISTANCE OF ERYTHROCYTES (50 PER CENT HEMOLYSIS PLOTTED)

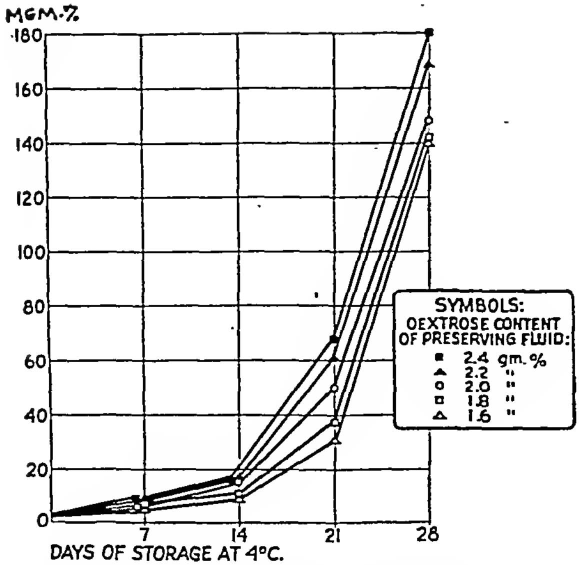

FIG. 3. EFFECT OF DEXTROSE CONCENTRATION ON HEMOGLOBIN IN THE SUPERNATANT PLASMA

week. The higher dextrose concentration showed a greater hemolysis (see Figure 3).

From these studies *in vitro* it was concluded that the amount of dextrose could be profitably reduced. Accordingly the following formula was adopted (February 1945) for further clinical trials:

B.M.H.—A.C.D. No. 3

Sodium citrate (trisodium, dihydric)	1.6 grams
Citric acid (monohydric)	0.56 gram
Dextrose (anhydrous)	1.5 grams
Water	75 ml.

Seventy-five ml. of this solution are placed in each collecting bottle and steam sterilized at 120° C. for 20 minutes. The pH of this solution is 4.8 and the pH of the resulting plasma varied from 7.05 to 7.45 (see Table I).

This formula was compared with the B.M.H.—A.C.D. No. 2 in a series of studies *in vitro*. It was noted that with the B.M. No. 3 the mean corpuscular volume remained within the normal range for at least 28 days, whereas with the B.M. No. 2 the tendency was to swelling beyond normal much sooner.

TABLE I

B.M.H. No. 3

A.C.D. plasma (75 ml.)

No. of bleedings	Pool No.	Days preserv. blood average	Blood average	Hb.	Prothrom. normal	Protein	pH	Turbidity filter 66	Complement titration						
									1/2	1/4	1/8	1/16	1/32	1/64	1/128
			ml.	*mgm. per cent*	*per cent*	*grams per cent*									
12	339A	4.3	454	2.6	78	6.1	7.41	107	−	−	−	±	+	++ ++	++ ++
13	339B	8.8	396	3.8	80	6.4	7.20	72	−	−	−	−	++ +	++ ++	++ ++
12	339D	8.2	430	2.9	80	6.2	7.30	72	−	−	−	−	++ +	++ ++	++ ++
13	341A	8	396	3.4	78	6.1	7.20	76	−	−	−	−	+	++ +	++ ++
11	341B	9.7	436	3.7	72	6.2	7.05	66	−	−	−	−	+	++ ++	++ ++
13	341D	8.5	458	3.0	77	6.2	7.20	69	−	−	−	−	+	++ +	++ ++
11	345A	8	414	3.8	77	6.3	7.40	68	−	−	−	−	+ −	++ +	++ ++
11	345B	10	436	2.9	78	6.4	7.40	63	−	−	−	−	+ −	++ +	++ ++
11	345D	3.2	395	3.9	70	6.2	7.45	69	−	−	−	−	+ −	++ +	++ ++
12	346	8	475	4.0	74	6.1	7.30	67	−	−	−	−	+ −	+	++ ++

A comparison of results of the studies of the osmotic resistance with the 2 solutions shows (see Figure 4) that the blood preserved in the B.M. No. 3 with lower dextrose content, remained within the normal limits for at least 35 days whereas , with the B.M. No. 2, abnormal resistance developed by the eighteenth day.

IN VIVO STUDIES

Radioactive iron

Radioactive iron studies on the survival of transfused erythrocytes after varying lengths of storage at 4° C. in B.M.—A.C.D. No. 3 have been reported elsewhere (2). The study showed optimal preservation up to at least 22 days, the survival of the erythrocytes being 84 per cent, a result better than that obtained with any other preserving fluid tested.

Hyperbilirubinemia

Determinations of the recipient's serum bilirubin level were carried out immediately before and 3 hours after completion of the transfusions. Figure 5 shows the result of such studies in 48 transfusions. The volume of transfusions was 450 to 500 ml. of whole blood (not including the preserving fluid).

While the data are as yet insufficient to allow a definite conclusion to be reached, there appeared to be no increase in the rate of hyperbilirubinemia during the first 21 days of storage.

Clinical observations

From 2/7/45 up to date, 5,010 specimens of blood have been collected in the Bryn Mawr A.C.D. No. 3. Studies of 1,215 whole blood transfusions were made and the reaction rate as well as the expected clinical results have been found to be optimal. No difficulties were encountered in the filtration of blood preserved up to 22 days. A complete description of a type of filter suitable for whole blood is given elsewhere (6). Proper filtration is obtained with finely woven nylon, stainless steel cloth (100 mesh) or multiple layers of more loosely woven gauze. When employing ordinary 40-mesh gauze, it should be used in 4 thicknesses.

To allow rapid filtration of blood, the filtering surface with any of the above mentioned materials should be 30 to 38 sq. cm.

It is essential to maintain the blood in constant slow rotating motion while it is being collected (7) and to mix the blood thoroughly before administration by gently rotating the bottle held at various angles for at least 3 minutes.

An objection was raised concerning the possibility that with lowered dextrose content the glycolytic action of the erythrocytes would deplete the dextrose before the estimated period of maximal

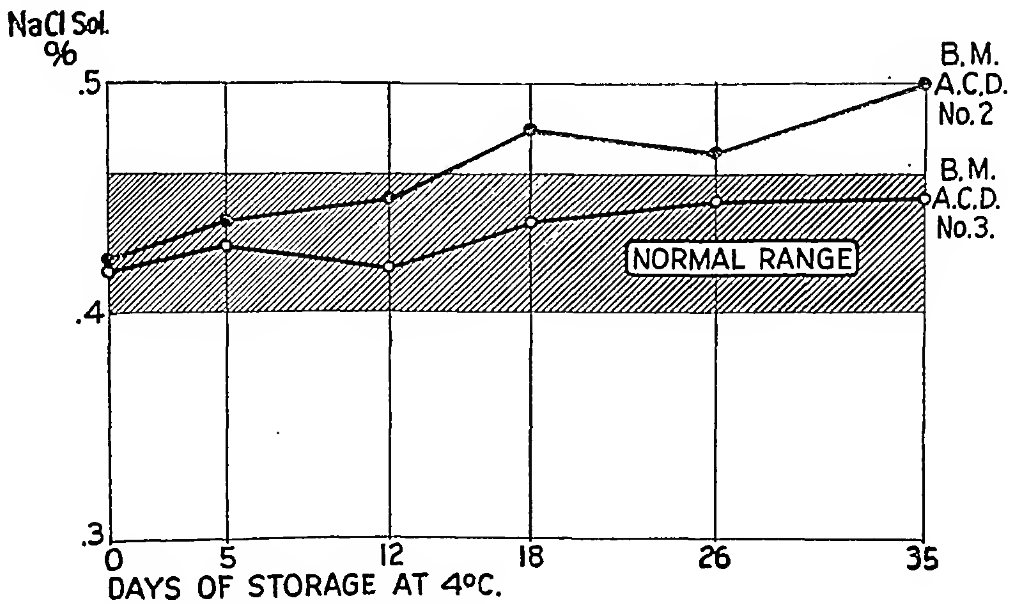

FIG. 4. OSMOTIC RESISTANCE OF ERYTHROCYTES STORED IN BRYN MAWR A.C.D. NO. 2 AND NO. 3

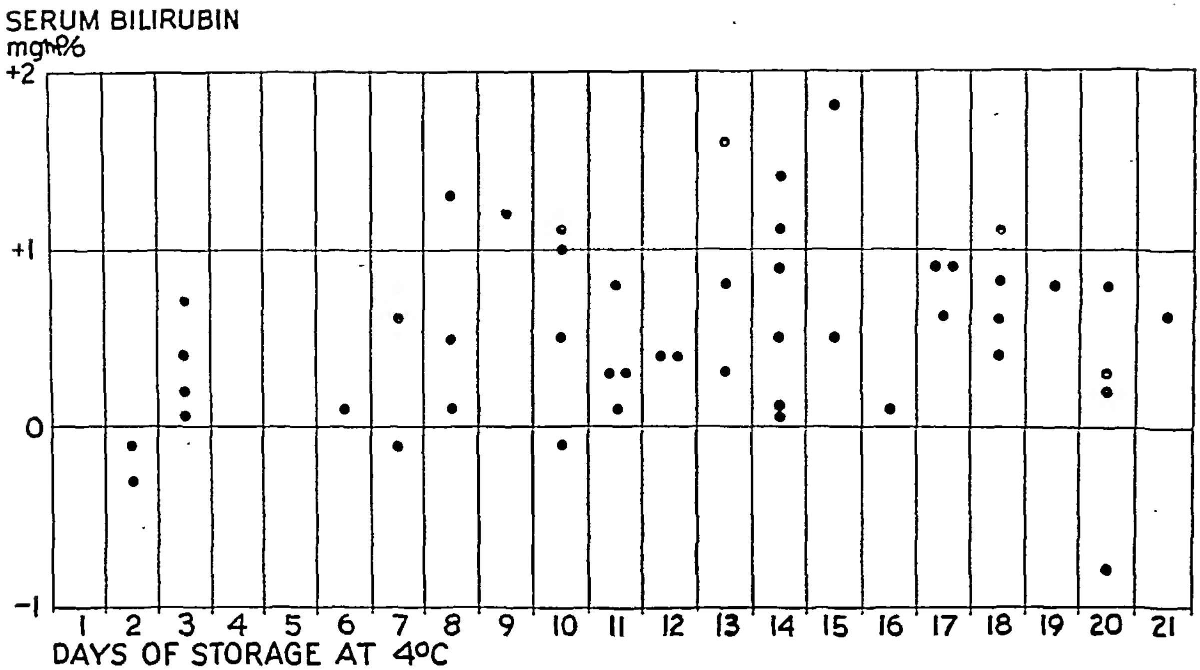

FIG. 5. VARIATIONS IN SERUM BILIRUBIN CONCENTRATION IMMEDIATELY BEFORE AND 3 HOURS AFTER TRANSFUSION OF BLOOD STORED IN A.C.D. B.M. No. 3

preservation of 4 weeks. Repeated studies have shown that such is not the case. For the determination of the dextrose content of whole blood and plasma, samples of blood were taken as described previously. A protein-free filtrate of whole blood and of plasma was prepared by the Folin-Wu technic and the dextrose content was determined by the Benedict method. The dextrose content of the red cells was calculated by differential.

Dextrose appears to be present in optimal concentration in both plasma and erythrocytes for at least 28 days (normal content of dextrose in plasma 95 mgm. per cent and in erythrocytes 66 mgm. [see Figure 6]).

These studies were repeated in a series of random specimens of blood obtained from the blood bank. The data contained in Figure 7 were obtained from determinations done on routine blood bank specimens, varying in volume from 450 to 500 ml. of whole blood. These were collected in the B.M.—A.C.D. No. 3, chilled and preserved undisturbed at 4° C. The dextrose determinations were done on an aliquot of well mixed blood obtained at the time of delivery of blood for administration to patients. The determinations were carried out by the Benedict method.

Figure 7 shows that for preservation up to 21 days at least 190 mgm. or more of dextrose are present.

A.C.D. PLASMA

The general composition of 10 pools of plasma obtained from blood preserved in B.M.—A.C.D. No. 2 were studied. When the findings were compared with similar ones obtained from the study of lots of plasma similarly prepared from plain citrated blood, it was found that the B.M.—A.C.D. No. 2 plasma had a lower hemoglobin content, a lower prothrombin content, a lower complement content, a lower protein content, a lower pH, and a lower turbidity. It is important to keep in mind that the A.C.D. blood was stored before separation of plasma for a period of time comparable to that of the plain citrated blood but that the dilution was somewhat greater. It was also found that the fibrinogen in the A.C.D. No. 2 plasma was definitely more unstable than in the ordinary citrated plasma. Flocculation of fibrinogen occurred more readily in plasma kept in the liquid state or thawed from the frozen state. A very considerable improvement in the quality of plasma was obtained by using the B.M.—A.C.D.

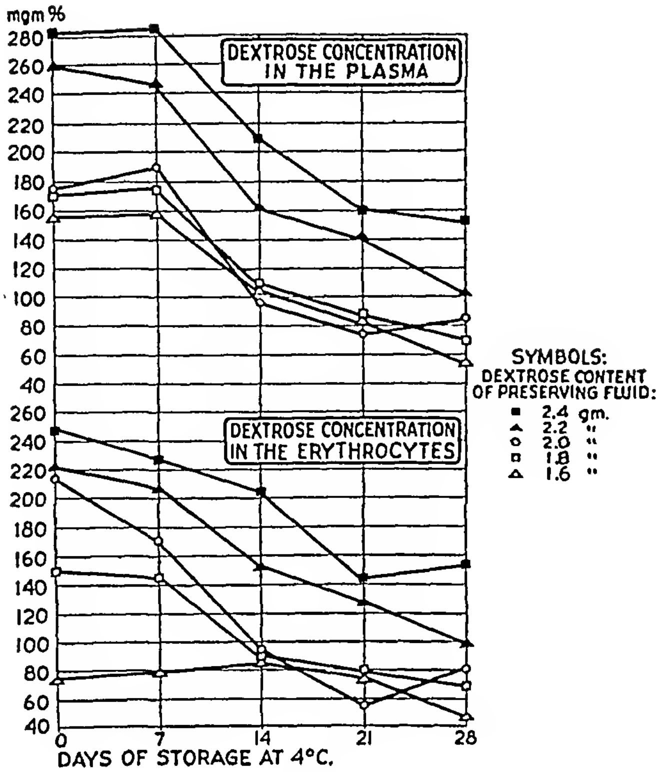

FIG. 6

No. 3 solution for the preparation of plasma. The general composition of 10 pools of such plasma is given in Table I.

When these findings were compared with those from lots of plasma similarly prepared from the B.M.—A.C.D. No. 2 solution (see Table II) it

TABLE II

Comparison of plasma obtained with A.C.D. solutions

	B.M.H. No. 2 A.C.D. sol. 100 *ml.*	B.M.H. No. 3 A.C.D sol. 75 *ml.*
Total no. of bleedings	110	119
Average time of preservation at 4° C., *days*	8.8	8.7
Average volume of bleedings, *ml.*	465	429
Hemoglobin, *mgm. per cent*	5	3.4
Prothrombin, *per cent of normal*	61.5	76.4
Protein, *grams per cent* (actual)	5.2	6.2
Protein, *grams per cent* (calculated)	5.1	5.7
Turbidity filter 66	74.9	72.9
pH	6.84	7.29
Complement titration	Comparable	

was found that the plasma obtained with the B.M. —A.C.D No 3 solution had lower free hemoglobin, substantially higher protein and prothrombin content, and comparable turbidity and complement titration although the average size of the bleeding was 36 ml. (8 per cent) less.

From Table II it is noted that the actual protein concentration is somewhat higher than one calculated from simple dilution factors. This is probably due to the fact that water enters the red cells whereas the protein does not, so that there is some concentration of proteins in the plasma. In other words the increase in concentration of the plasma protein is due to the increased hematocrit of the red cells. The pH of the plasma from the B.M.—A.C.D. No. 3 averages 7.29 instead of 6.85 of the previous solution. This change was responsible for the improved prothrombin preservation. It was also noted that when the plasma obtained with the use of the B.M.—A.C.D. No. 3 was frozen and thawed, according to the proper procedure outlined elsewhere, the fibrinogen was fully as stable as that obtained from the plain citrated blood. This optimal stability of fibrinogen is conditional on proper mixing of the blood and preserving fluid during the collection. The necessity of proper mixing of blood with the preservative solution cannot be sufficiently emphasized. This mixing must be done by a gentle continuous ro-

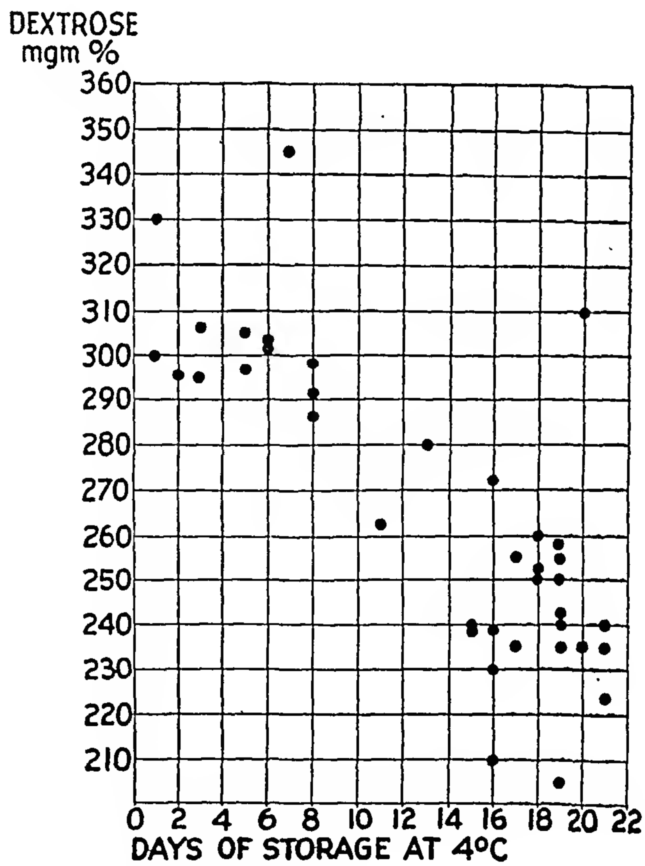

FIG. 7. DEXTROSE CONCENTRATION IN WHOLE BLOOD STORED AT 4° C. IN A.C.D. SOLUTION (B.M. No. 3)

tating motion. This is best accomplished by the use of a mechanical rotator, which does away with the unavoidable irregularities of the hand mixing and with the necessity of having a person in attendance for each patient. The rotators provided by the A. H. Thomas Co. of Philadelphia have been found excellent when fitted with a suitable heavy wooden bottle holder. Extensive studies have shown that the plasma obtained with the B.M.—A.C.D. No. 3 solution can be preserved in the frozen state or dried from the frozen state with results in all ways comparable with those obtained when a plain citrated plasma is used. It is to be noted here that when plasma prepared from whole blood preserved in a solution containing a higher glucose content is dried, the period of drying is lengthened because of the necessity of maintaining the plasma at a lower temperature while the water is being sublimated. When regenerating plasma prepared from A.C.D. blood, the use of a .1 per cent citric acid solution exerts the same beneficial effect in the preservation of prothrombin which has been noted for the plain citrated plasma (8).

CONCLUSIONS

An acid citrate-dextrose solution is proposed which has given good results for preservation of whole blood and yields a good stable plasma. This A.C.D. solution has a smaller volume and a lower dextrose concentration than solutions previously used. This solution has proved entirely satisfactory in practical use in over 5,000 blood collections.

BIBLIOGRAPHY

1. Loutit, J. F., Mollison, P. L., and Young, I. M., Citric acid-sodium citrate-glucose mixtures for blood storage; report to Medical Research Council from Southwest London Blood Supply Depot. Quart. J. Exper. Physiol., 1943, 32, 183.
2. Strumia, M. M., Blake, A. D., and Wicks, W. A., The preservation of whole blood. J. Clin. Invest., 1947, 26, 667.
3. Bulletin of the Subcommittee on Blood Substitutes, January 19, 1945 meeting, Washington, D. C.
4. See technical note at end of paper.
5. Parpart, A. K., Lorenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, A. M., The osmotic resistance (fragility) of human red cells. J. Clin. Invest., 1947, 26, 636.
6. Strumia, M.M., Filter-drip unit for dispensing blood and plasma. Am. J. Clin. Path., Tech. Sect., 1944, 8, 75.
7. Strumia, M.M., McGraw, J. J., and Reichel, J., The preparation and preservation of human plasma. I. Collection of blood and separation of plasma. Am. J. Clin. Path., 1941, 11, 175.
8. Strumia, M. M., Preservation of prothrombin in dried plasma. J. A. M. A., 1942, 119, 710.

TECHNICAL NOTE

DETERMINATION OF HEMOGLOBIN IN PLASMA (VISUAL COLORIMETRY)

By W. G. Karr and F. W. Chornock

SOLUTIONS:

Acetic acid—10 per cent:

Pipette 10 ml. glacial acetic acid into a 100 ml. volumetric flask and dilute to mark.

Benzidine dihydrochloride:

Dissolve 140 mgm. of benzidine dihydrochloride in about 200 ml. of water with warming. Cool, add 3.3 ml. of 10 per cent acetic acid and dilute to 500 ml. in a volumetric flask. Keep in a brown bottle in the refrigerator. (The acidity of this reagent is correct only for use with plasma as directed below.)

Hydrogen peroxide—1 per cent:

Dilute 0.5 ml. of Merck 30 per cent Superoxol to 15 ml. in a graduate. Prepare this solution just before use.

Artificial standard:

Ferric ammonium sulfate—1 per cent.
Potassium ferrocyanide —1 per cent.
Gum ghatti —1 per cent.

The iron and the ferrocyanide solutions are prepared accurately by careful weighing and dilution in a volumetric flask. Select clean, purple crystals of ferric ammonium sulfate. The gum ghatti is made approximately by suspending 1.5 grams on a wire or cloth gauze in 100 ml. of cold water and allowing it to dissolve overnight. If necessary, filter through cheesecloth to remove particulate matter.

In a liter volumetric flask, pipette exactly 13.0 ml. of the 1 per cent potassium ferrocyanide. Dilute to about 900 ml. Add 10 ml. of 1 per cent gum ghatti.

Add 20 ml. of the 1 per cent ferric ammonium sulfate. Dilute to mark. Mix. Keep in ice box.

Color is equivalent to 25 mgm. Hb per 100 ml. when the color is developed as in Method described below.

Serial standards:

Prepare 250 ml. of each of the following dilute standards by diluting the artificial standard with water as indicated.

Water	Standard	Hb. equiv. (C) mgm. per 100 ml.
0	250	25
50	200	20
100	150	15
150	100	10
200	50	5

Mix and place in 250 ml. bottles. Keep in ice box.

METHOD:

Dilute 1 ml. of plasma to 10 ml. with water. Measure 4 ml. of the dilute plasma into a test tube. Add 15 ml. of the benzidine dihydrochloride reagent. Add 1 ml. of the 1 per cent hydrogen peroxide and mix. Immerse in an ice bath for 35 minutes. At the end of this time select the artificial standard which is nearest the color of the unknown plasma. Pour 20 ml. into a test tube and add 0.4 ml. of the original undiluted plasma. Compare in the colorimeter.

If the blue color developed is appreciably greater than the 25 mgm. standard, the determination should be repeated using 4 ml. of a dilution of plasma greater than 1 to 10. Make appropriate change in the calculation.

CALCULATION:

X = mgm. Hb. per 100 ml. plasma.
S = reading of standard on the right.
U = setting of the unknown on the left.
C = equivalent Hb. concentration of the artificial standard selected for reading.

$X = \frac{S}{U} \times C$. Set U at 10. $X = \frac{S \times C}{10}$.

Blank: 4 ml. diluted plasma plus 15 ml. benzidine reagent plus 1 ml. water in icebath with unknowns.

THE *IN VITRO* PRESERVATION AND POST-TRANSFUSION SURVIVAL OF STORED BLOOD[1]

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INTRODUCTION

The therapeutic value of blood transfusion is well established and experiences during World War II demonstrated the urgent need for readily available supplies of whole blood. Blood plasma and plasma "substitutes," although effective in combatting shock and in replacing plasma lost into burned and traumatized areas, cannot restore erythrocytes lost from hemorrhage or intravascular destruction (1).

Civilian and military experiences have demonstrated the practicability and value of whole blood stored or "banked" in advance of need, and immediately available for use in any emergency. In civilian practice, the blood bank has greatly facilitated the procedure of blood transfusion and has made feasible the routine use of whole blood transfusion. In military practice, banked blood is an absolute essential since it is impractical to bleed donors for transfusion under combat conditions, but it is under these very conditions that whole blood is most urgently needed.

Although stored blood is highly desirable practically and economically, to be effective, the viability of the erythrocytes must be maintained during the period of storage since the major purpose of transfusing whole blood is to supply the recipient with functional erythrocytes. Erythrocytes destroyed immediately after transfusion not only are valueless to the recipient, but the intravascular liberation of large amounts of hemoglobin from destroyed cells actually is deleterious and dangerous to the recipient. The life span of the erythrocyte *in vivo* is between 100 and 120 days but after removal from the circulation the period of viability is greatly reduced. Unless special precautions are observed, blood is unsatisfactory for transfusion purposes after storage for periods as brief as 3 to 5 days (2).

Attempts to preserve the functional capacity of stored erythrocytes were first made by Rous and Turner in 1916 (3) with considerable success. Robertson, an English army surgeon, using the solution devised by Rous and Turner, made the first successful use of bank blood during the first World War (4). The solution of Rous and Turner had several serious shortcomings, however, and did not receive general use. During the ensuing 20 years various modifications of the Rous and Turner solution were made and many new types of preservative solutions were devised (5 to 11). None of these preservatives was entirely satisfactory, however, and little exact information was presented as to how effective each actually was in maintaining the viability of the stored cells.

Under the stimulus of World War II various English (12 to 16) and Canadian (17, 18) workers reinvestigated the entire subject of blood preservation, and used as the criterion of effective preservation the post-transfusion survival of erythrocytes determined with the differential agglutinating techniques of Ashby (19) and of Wiener (20). These agglutinating techniques allow a qualitative comparison of the effectiveness of various preservatives, and also make possible the determination of the total duration of time that transfused cells survive in the recipient's circulation. As usually performed, however, these methods do not permit an accurate quantitative evaluation of the *immediate* post-transfusion erythrocyte survival; yet it is this immediate survival that is most

[1] The work described in this paper was done under contracts recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Massachusetts Memorial Hospitals and the Massachusetts Institute of Technology.

[2] Formerly Welch Fellow in Internal Medicine, National Research Council. These investigations were carried on while under tenure of the Welch Fellowship in Internal Medicine.

important in the majority of transfusions. In general, however, the findings and conclusions of the English investigators have been similar or identical to ours which were obtained with a very different, and, we believe, more exact technique.

To determine the most effective method of preserving the viability of erythrocytes in stored blood, we have investigated 16 preservative solutions. We have judged the effectiveness of these solutions from the standpoint of the post-transfusion survival of erythrocytes stored for varying lengths of time in each solution, and we have determined the post-transfusion survival of red blood cells by the radioactively labeled erythrocyte technique previously described by Ross and Chapin (2). In an attempt to determine which factors in the preservative medium promoted cell survival, extensive studies of the changes in physical and chemical properties of the stored blood were made in conjunction with the survival studies. It soon became apparent to us, as it has to others (21, 22), that the *in vitro* characteristics of stored blood are of little value in predicting the suitability of stored blood for transfusions. The really significant criterion of the value of a blood preservative is how well it maintains the viability of the stored erythrocytes, and this attribute can be judged only by studying the survival of the cells after transfusion into a recipient.

PROCEDURES AND METHODS

The radioactively labeled erythrocyte technique of evaluating the survival of erythrocytes following transfusion has been outlined in a previous paper (2). In brief, this method consists of administering orally or parenterally one of the radioactive isotopes of iron to a prospective blood donor whose hematopoietic system incorporates the radioactive iron in the hemoglobin molecules of newly formed erythrocytes. These erythrocytes are thus "labeled," since once an atom of radioactive iron is built into the hemoglobin molecule it does not exchange with the nonradioactive iron of the plasma or tissues. It remains within the erythrocyte during its lifetime and is liberated from the red cell only when the red cell is destroyed (23). Hemoglobin and erythrocytes containing this radioactive material differ in no physiological respect from unlabeled cells, but by appropriate physical techniques (24, 25) they can be accurately detected when present in dilutions as great as 1 in 3,000.

When donor cells labeled with radioactive iron are transfused into compatible recipients, they rapidly mix with the recipient's cells but still can be detected quantitatively in samples of the recipient's blood. If the labeled cells are destroyed after transfusion, there is a decrease in the concentration of radioactivity in the recipient's blood, and samples taken consecutively will reveal the extent and rate of cell destruction. The effectiveness of various blood preservatives can thus be evaluated by observing their influence on the survival of transfused radioactively tagged erythrocytes.

Preparation of donors of radioactively labeled erythrocytes

In evaluating the effectiveness of preservative solutions we believe that considerable importance should be attached to procuring uniformity in the donor blood. Individual variations in the bloods of different donors may markedly influence the storage and transfusion properties of the donor's erythrocytes and give erroneous impressions of the preservative solutions under investigation. To eliminate the factor of donor-cell variation from our studies all bloods used in our experiments were procured from a single blood donor with the exception of a series of control observations when blood was obtained from a second donor. The behavior of the cells of these 2 donors was identical. Both donors were healthy young adult males with normal erythrocyte, hemoglobin, and leukocyte characteristics. The blood of donor I, on whom most of the observations were made, was Group O, Rh factor negative. Donor II provided blood for control observations. His blood was Group O, Rh factor positive.

The radioactive isotope of iron, Fe^{55}, was administered intramuscularly or intravenously in an aqueous solution of iron and ammonium citrate. Donor I received 2 series of injections. In the first series a total of 4.68 mgm. iron with an activity of 7,000,000 counts per minute given in 20 injections during a period of 71 days. second series of injections was administered 8 mon later when 4.36 mgm. of iron with an activity of 6,610,(counts per minute were given over a period of 74 d The activity is expressed as the absolute number of co determined with a beryllium window Geiger-M counter (25). The radioactive iron was rapidly in porated in the newly formed hemoglobin and erythroc of the donors. Although the rate of incorporation i hemoglobin and the percentage utilization of the injec radioactive iron are considered in a separate publicati we may point out that approximately 60 per cent of given dose of injected radioactive iron appeared in the circulating erythrocytes of the donors and that maximum utilization of a single dose was complete in approximately 20 days. The maximum specific radioactivity produced in the donor's blood amounted to 4,180 counts per minute per ml. of erythrocytes, 12,550 counts per minute per gram of hemoglobin, or 3,760 counts per minute per mgm. of hemoglobin iron.

Since the labeling radioactive iron first becomes available to the donor for hemoglobin synthesis at the time of the first injection of the isotope, the maximum age of the donor erythrocytes is determined by the interval of time elapsing between the first injection of radioactive

iron and the times that the donor is venesected and the blood transfused. Thus, it is possible to state definitely that the labeled cells of a given transfusion have a maximum age of 7 days, 30 days, 100 days, etc. As previously described (2), radioactive iron liberated from destroyed radioactively labeled erythrocytes is rapidly reutilized by the body and incorporated into newly formed hemoglobin and erythrocytes. Therefore, 4 to 6 months after the first injections of radioactive iron the labeled cells are representative of the mixed age population of the whole cell mass. These considerations are important in evaluating the behavior of young, old, and mixed age cells during storage and after transfusion.

With the exception of the previously reported (2) observations on blood stored in tri-sodium citrate solutions the donor erythrocytes used for these studies varied in possible maximum age from 80 days to 120 days. The majority of observations were made on bloods in which the radioactive labeled cells were of all ages and were representative of the age distribution of the entire erythrocyte population.

Technique of collection and storage of radioactively labeled donor blood

After a fasting period of at least 12 hours the donors were bled from an antecubital vein through a closed system into the preservative solution under investigation. Except when noted to the contrary, 400 to 500 ml. of blood were drawn into the preservative solution contained in a 600-ml. pyrex Fenwal transfusion bottle, both solution and container previously having been chilled to 4° C. The blood was stored immediately in the dark in a stationary mechanical refrigerator thermostatically regulated 4° to 6° C. until aliquots were removed for transfusion.

Recipients of transfusions

All recipients were normal healthy adult male or female medical students, internes, or hospital technicians. The of normal individuals excluded the uncontrollable factor of effect of disease processes in the recipient on the post-transfusion survival of erythrocytes. All donors and recipients used in these studies were of blood group O. Most of the studies were performed with Rh negative (rh, rh or cde, cde) blood. In experiments in which Rh positive blood was employed, only Rh positive recipients were used. Careful cross-matching with the Landsteiner test tube technique verified complete compatibility between donor and recipient bloods in all cases.

We believe that the use of donor blood of the same blood group as that of the recipient is important in these studies, since it is now well established that the iso-agglutinins contained in transfused blood may destroy the recipient's own erythrocytes (26). If this should occur following a transfusion of radioactively labeled blood the specific radioactivity of the recipient's erythrocytes would be increased and a false impression of the post-transfusion survival would be obtained.

Techniques of transfusion and sampling of recipients

All transfusions were given early in the morning while the recipient was in a fasting state. The recipient remained in bed during the course of the injection of the blood and for 1 hour thereafter, and then resumed full activity.

Aliquots of radioactively labeled blood for transfusion and for hematologic, chemical, and radioactive studies were removed by aspiration from the storage flasks after the cells and plasma had been gently and thoroughly mixed by rotation. The storage flask was then returned to the refrigerator. Immediately after removal from the storage flask a known amount, usually 100 ml., of the chilled blood was injected into the recipient through a No. 18 needle from a calibrated 100-ml. syringe. Two to 4 minutes were required to make the injection. A known amount of the dye T–1824 was then immediately injected through the same needle into the vein of the recipient for purposes of plasma volume determination.

Samples of blood for hematologic study and for radioactivity and T–1824 concentration determinations were subsequently removed through a No. 18 needle in the opposite arm. A portion of the sample was placed in the mixed oxalate of Heller and Paul (27) for hematologic study, and the remainder of the sample was placed in a calibrated conical centrifuge tube and allowed to clot. The serum from the clotted blood was used for spectroscopic study and for determination of T–1824, hemochromogen, and bilirubin concentrations. The red blood cells from a known volume of oxalated and clotted blood were digested for radioactivity determinations as subsequently described. The exact volume of red blood cells and the amount of hemoglobin in each sample were calculated from the volume of blood and the hematocrit and hemoglobin values determined on an aliquot of this blood.

Ninety transfusions of blood were given in this fashion. There were no reactions of any sort in 88 of the subjects. Two subjects experienced a mild febrile reaction which was proved to be caused by the lot of T–1824 dye solution used for the blood volume determination in these 2 subjects.

Radioactivity determinations

The specific radioactivity of the donor's and recipient's whole blood, erythrocytes, hemoglobin, and hemoglobin iron was determined with techniques similar to those described by one of us in previous papers (2, 24), with some modifications (25). In these experiments we have used the radioactive isotope Fe^{55} (half life approximately 5 years) instead of the radioactive isotope Fe^{59} (half life 47 days). The longer half life of Fe^{55} makes this isotope much more satisfactory than Fe^{59} for long-term cell tracing experiments. The radiations from Fe^{55} are low energy x-rays and have necessitated the use of a beryllium window counting tube (25) instead of the thin mica window tube previously used. The preparation of the samples of blood for determination of radioactive con-

tent by acid digestion, neutralization, and precipitation of iron is identical with those previously described (24) with the exception that the iron in the present series of experiments was precipitated as ferric hydroxide instead of ferrous sulfide.

To eliminate variation in self-absorption the iron content of each sample was determined by multiplying the grams of hemoglobin in the sample by 0.00334 (that fraction of hemoglobin which is iron) and then enough non-radioactive iron was added to the sample to make its total iron content 10 mgm.

The iron was electrolytically deposited in a uniform, thin layer on copper discs as previously described (24, 25), but the plating process was carried out from a ferric rather than a ferrous solution. This required a longer period of electroplating than was the case when a ferrous solution was employed but was satisfactory for quantitative removal of the iron.

An aliquot of the donor blood which had been injected into each subject was assayed for radioactivity with the radioactivity determination of each sample of the recipient's blood, thus eliminating the necessity of making corrections for the decay of the radioactive iron, counting tube variations, etc.

Determination of plasma, blood and erythrocyte volumes

The determination of the post-transfusion survival of erythrocytes by any method is no more accurate than the accuracy with which the total circulating erythrocyte volume of the recipient is determined. This is true not only of the radioactively tagged cell method but also of the Ashby (19) and Wiener (20) methods of differential agglutination. The critical significance of the total erythrocyte volume will be evident from consideration of the following formula for calculating the post-transfusion survival of radioactively labeled red blood cells.

$$\left.\begin{array}{c}\text{Per cent of transfused cells}\\ \text{surviving in the recipient's}\\ \text{circulation}\end{array}\right\} = \frac{\begin{array}{c}\text{Radioactivity per}\\ \text{ml. of recipient's}\\ \text{cells}\end{array} \times \begin{array}{c}\text{Recipient's red}\\ \text{blood cell}\\ \text{volume}\end{array} \times 100}{\text{Total radioactivity of transfused blood}}$$

Although the radioactivity concentrations of the recipient's and donor's bloods can be determined with considerable accuracy, the method of determination and interpretation of the recipient's red blood cell volume is the subject of considerable difference of opinion. A discussion of the latter problem is beyond the scope of this paper, but it seems worthwhile to point out that the neglect of the significance of the cell volume, the assumption that it represents a constant per cent of the body weight or surface area, or its estimation purely on the basis of the plasma volume and the uncorrected venous hematocrit will give grossly erroneous values for the survival of transfused cells. In the differential agglutinating technique of Ashby or Wiener, calculations of survival are based on the assumptions that all of the transfused cells are surviving at the conclusion of the transfusion and that the number of non-agglutinated cells present in the recipient's blood at the conclusion of the transfusion represent 100 per cent survival of the transfused cells. This assumption is not valid, since our data indicate that many transfused erythrocytes are removed from the recipient's circulation very rapidly (actually during the course of the transfusion). This is particularly true when bloods with poor survival characteristics are transfused. The poorer the post-transfusion survival of stored blood the more rapidly the non-viable cells are removed from the circulation. Because of this fact most of the studies performed with modifications of the Ashby technique have indicated erroneously high post-transfusion survival.

Calculation of the cell volume from the plasma volume and venous hematocrit repeatedly has been shown to give values 15 to 30 per cent too high, unless correction is made for the error of the hematocrit as determined by centrifugation and for the unequal distribution of plasma and cells throughout the vascular system (28 to 31). Unless allowance is made for these errors, the calculated survival of labeled erythrocytes after transfusion will be 15 to 30 per cent too high.

Realizing that determinations of post-transfusion erythrocyte survival and evaluation of preservative fluids and techniques were so critically affected by the red blood cell volume we have determined this volume in an identical fashion in each recipient and have determined it with a technique which we believe represents a conservative factor in evaluating the post-transfusion erythrocyte survival. Although our method may represent too small a cell volume and, therefore, a low value for post-transfusion survival, it certainly does not give too high a cell volume or indicate too high a post-transfusion survival.

The recipient's red blood cell volume was determined as follows:

1. The plasma volume was determined with the dye T-1824 according to the method of Gibson and Evans (32), samples being taken at 15, 25, 30, 35, and 60 minutes, and the dye concentrations being determined on serum in the micro unit of the Evelyn colorimeter.

The L values obtained at 620 millimicrons were corrected with a reading at 540 millimicrons to correct for the effect of serum hemoglobin (which was present in the serum following transfusion of blood with poor survival characteristics). The L values were also corrected for fluctuation in plasma protein concentration (33, 34). The disappearance curve was extrapolated to the time of injection.

2. The blood volume and cell volume were calculated from the following formula, employing the corrected hematocrit value previously described (28):

$$\text{Blood volume} = \frac{\text{plasma volume}}{1 - (\text{hematocrit} \times 0.92)}$$

$$\text{Cell volume} = \text{blood volume} - \text{plasma volume.}$$

3. The cell volume so determined was corrected to compensate for the error introduced by unequal distribution of erythrocytes and plasma throughout the vascular system (29).

$$\text{Corrected cell volume} = \text{cell volume} \times 0.85.$$

The cell volumes of normal human subjects determined in this fashion compare very closely with the cell volume determined in the same subjects with the use of radioactive tagged cells (31).

We again wish to emphasize the fact that the use of the cell volume determined in this fashion provides a conservative estimate of post-transfusion erythrocyte survival and allows an accurate comparison of various preservative fluids.

Calculation of post-transfusion survival

The radioactivity present in the circulating red blood cell mass of the recipient was determined at various intervals following the transfusion by applying the formula:

$$\left.\begin{array}{c}\text{Per cent of transfused radio-}\\ \text{activity present in the recipient's}\\ \text{circulation}\end{array}\right\} = \frac{\begin{array}{c}\text{Radioactivity per}\\ \text{ml. of recipient's}\\ \text{red blood cells}\end{array} \times \begin{array}{c}\text{Recipient's red}\\ \text{blood cell}\\ \text{volume}\end{array} \times 100}{\text{Total radioactivity of the transfused blood}}$$

The specific radioactivity of the recipient's cells and thus the percent of transfused radioactivity present in the recipient's circulation decreased following the transfusion, the lowest values being reached at the end of approximately 24 or 48 hours. Subsequently, the concentration of radioactivity progressively increased (Figures 1 and 2). We have interpreted the decrease in radioactivity as indicating the removal of the non-viable transfused erythrocytes from the recipient's circulation. The rate of this removal was most rapid in those bloods showing the poorest post-transfusion survival. We have interpreted the increase in radioactive iron in the recipient's circulation as indicating the reutilization of the radioactive iron liberated from the destroyed transfused erythrocytes for the formation of new erythrocytes by the recipient. Support is given to this interpretation by observations of similar buildup curves following infusion of solutions of hemoglobin labeled with radioactive iron (35).

During the first 24 hours following the transfusion the amount of radioactive iron incorporated in newly formed recipient's erythrocytes is too small to contribute significantly to the total radioactivity in the recipient's circulation. Therefore, during this period all of the radioactivity in the recipient's blood can be attributed to the presence of surviving labeled donor red blood cells and indicates post-transfusion survival. After 24 hours, the reutilization of radioactive iron and its incorporation in the recipient's own erythrocytes may occur more rapidly than the removal of the transfused labeled cells and may be great enough to indicate a falsely high post-transfusion survival.

Because of these considerations the minimum specific radioactivity of the recipient's erythrocytes, which usually is reached 24 hours after transfusion, has been adopted as indicating the minimum post-transfusion survival of the labeled donor erythrocytes.

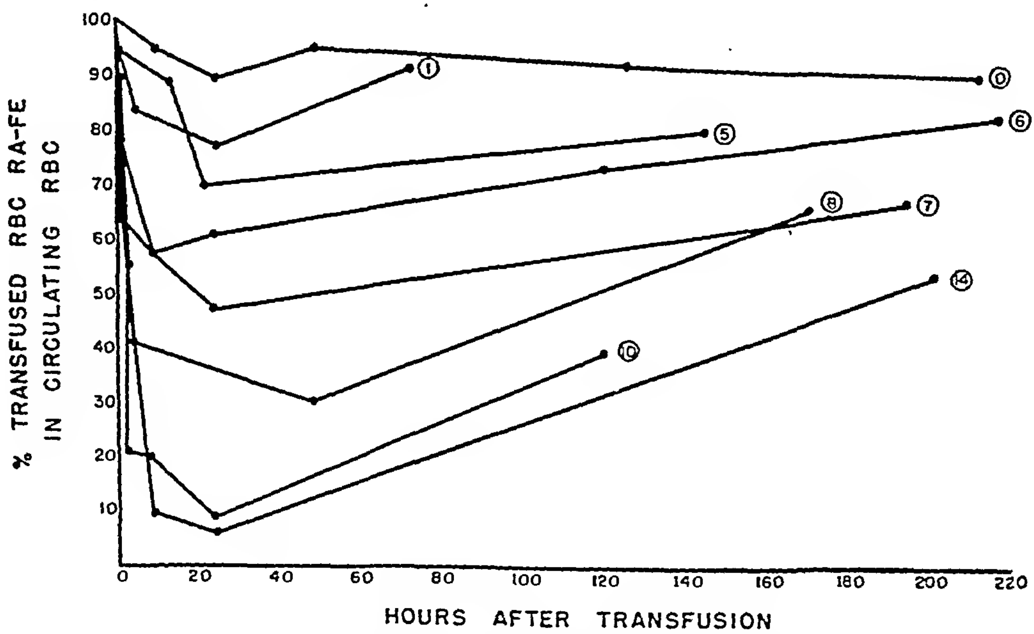

FIG. 1. THE EFFECT OF STORAGE AT 4° C. ON THE POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES OF WHOLE BLOOD STORED IN TRISODIUM CITRATE (SOLUTION NO. 1)

The circled figures indicate the number of days the blood was stored prior to transfusion.

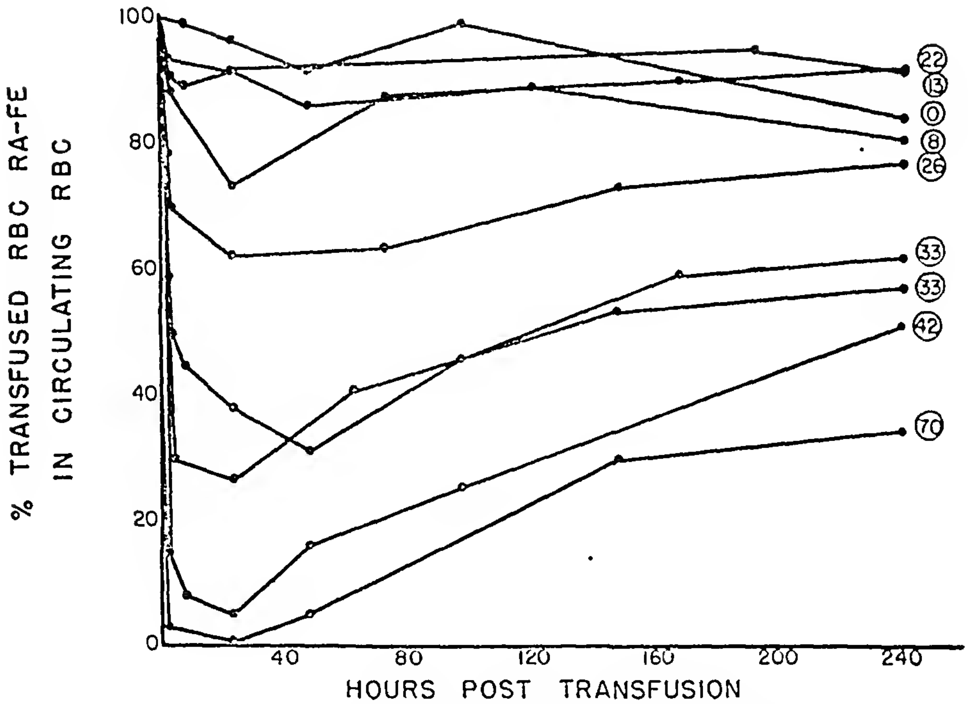

FIG. 2. THE EFFECT OF STORAGE AT 4° C. ON THE POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES OF WHOLE BLOOD STORED IN DENSTEDT'S SOLUTION (SOLUTION No. 2)

The circled figures indicate the number of days the blood was stored prior to transfusion.

This minimum survival has been used as the criterion for evaluating the effectiveness of the blood preservative under consideration.

Preservative fluids studied

All solutions (except the corn syrup and Alsever's solutions which were obtained commercially) were prepared with pyrogen-free, freshly distilled water and reagent chemicals. All glassware and storage bottles (except the Baxter bottles containing Alsever's solution) were pyrex glass. With the exception of a few experiments that were carried out at room temperature, all bloods were drawn into containers and solutions which previously had been chilled to 4° C. and were immedately stored at 4° to 6° C.

The fluids studied may be grouped into those used for the preservation of whole blood and those used for the preservation of erythrocytes separated from blood plasma. A description of the constitution, the methods of preparation, and use of these fluids follows.

A. PRESERVATIVE FLUIDS FOR WHOLE BLOOD

Solution No. 1: Trisodium citrate

Trisodium citrate (2.5 per cent)	50 ml.
pH of solution	7.5
Whole blood	450 ml.
pH citrate blood mixture	7.4

Nineteen transfusions were given of blood stored in this solution 0 to 14 days.

Solution No. 2: Denstedt's or McGill No. II solution

Isotonic sodium citrate (3.2 per cent)	80 ml.
Isotonic dextrose (5.4 per cent)	80 ml.
Isotonic phosphate buffer pH 7.4	40 ml.
Whole blood	400 ml.
pH of solution	7.4
pH solution-blood mixture	7.4

The citrate, dextrose, and buffer solutions were prepared and autoclaved separately, cooled, and mixed with aseptic precautions in sterile Fenwal bottles according to the method described by Denstedt, *et al* (17). Twelve transfusions were given of blood stored in this solution 0 to 70 days.

Solutions No. 3 and 4: Alsever's solutions No I and II[3] *(Baxter No. I and II)*

Solution No. 3—Alsever's solution No. I

Dextrose (U.S.P.)	2.05 per cent	of this solution—500 ml.
Sodium citrate (U.S.P.)	0.80 per cent	
Sodium chloride (U.S.P.)	0.42 per cent	
Citric acid (U.S.P.)	0.04 per cent	
Whole blood		500 ml.
pH of solution		6.78
pH of solution-blood mixture		7.5

[3] These solutions, modifications of the one described by Alsever and Ainsle (11), were prepared and provided by the Baxter Laboratories, Incorporated, through the courtesy of Dr. N. M. Nesset. The formulae given here were provided by the Baxter Laboratories as descriptive of their constitution.

Four transfusions of blood stored in this solution 1 to 36 days were given.

Solution No. 4: Alsever's solution No. II

Dextrose (U.S.P.)	2.05 per cent	of this solution	—500 ml.
Sodium citrate (U.S.P.)	0.80 per cent		
Sodium chloride (U.S.P.)	0.42 per cent		
Citric acid (U.S.P.)	0.055 per cent		
Whole blood			500 ml.
pH of solution			6.0
pH of solution-blood mixture			7.2

Four transfusions were given of blood stored in this solution 7 to 35 days.

Solution No. 5: Acid citrate-dextrose solution of Loutit and Mollison[4]

Disodium citrate (2 per cent)	100 ml.
Dextrose (15 per cent)	20 ml.
Whole blood	430 ml.
pH of solution	5.0
pH of solution-blood mixture	6.8

This solution was prepared according to the method described by Loutit and Mollison (14). Nine transfusions of blood stored in this solution 2 to 34 days were given.

Solution No. 6: Acid citrate-dextrose solution of reduced volume

Trisodium citrate	2.20 grams	of this solution	—75 ml.
Citric acid	0.80 grams		
Dextrose (anhydrous)	2.25 grams		
Water	100 ml.		
pH	5.0		
pH of preservative-blood mixture	6.8		
Whole blood			500 ml.

(15 ml. of this solution used for each 100 ml. of whole blood.)

In the proportions used, this solution provided approximately 2 grams of disodium citrate per 500 ml. of blood, and an eventual 24 millimolar concentration of citrate ion in the supernatant plasma of the preservative-blood mixture. In these respects, and in the pH of the solution and the solution-blood mixture, solutions No. 5 and 6 were identical. The dextrose content of solution No. 6 was approximately ½ that of solution No. 5, however, and the volume of the diluent was 62.5 per cent of solution No. 5.

Four transfusions of blood stored in this solution 6 to 28 days were given.

[4] The disodium citrate used in preparing this solution and others containing disodium citrate was kindly prepared and provided by Dr. Walter Clarke of the Eastman Kodak Company, Rochester, New York. In the event that crystalline disodium citrate should not be available a solution of disodium citrate can readily be prepared as follows:

Trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	1.662 grams
Citric acid ($H_3C_6H_5O_7 \cdot H_2O$)	0.595 grams
Water	100 ml.
pH	5.0

B. PRESERVATIVE FLUIDS FOR ERYTHROCYTES SEPARATED FROM THEIR PLASMA

The separated cells for solutions 7, 8, 9, and 10 were prepared as follows: 450 ml. of donor blood were drawn into 50 ml. of previously chilled 2.5 per cent trisodium citrate solution of pH 7.5. The cells were immediately sedimented by centrifugation at 2,500 r.p.m. for 1 hour, and the plasma then removed by aspiration with aseptic precautions. For the experiments with solutions 11, and 12, 400 ml. of blood were drawn into 100 ml. of 2 per cent disodium citrate solution of pH 5.0. Centrifugation and separation of plasma were carried out as previously described. The cell mass for solution 13 was obtained from defibrinated blood which was centrifuged and separated from its plasma as described above.

A description of the solutions and techniques used in the preservation of erythrocytes separated from their plasma follows:

Solution No. 7: Human albumin solution[5]

Human albumin	6.00 per cent	of this solution	—400 ml.
Dextrose (anhydrous)	1.00 per cent		
Sodium chloride	0.85 per cent		
Phosphate buffer (0.125 molar pH 7.4)	0.07 per cent		
Red blood cell mass			200 ml.
pH of solution			7.35
pH of solution-blood mixture			7.35

The solution was sterilized by passing it through a Seitz filter with pressure. It was chilled to 4° C., and the packed red blood cells were pipetted into it. The cells and preservative were mixed thoroughly then stored at 4° C.

Five transfusions of cells preserved in this fashion were given; the duration of storage varying from 10 to 46 days.

Solution No. 8: Corn syprup

Corn syrup (10 per cent)	200 ml.
Packed blood cells	200 ml.
pH of corn syrup	4.0
pH of corn syrup-blood mixture	6.7

This 10 per cent solution of sterile corn syrup was provided in ampules through the courtesy of Dr. William Thalhimer of New York City. The concentration of the corn syrup before it is diluted is stated to be (36):

Dextrose	17.7 per cent
Maltose	16.8 per cent
Higher sugars	16.2 per cent
Dextrins prosugars	29.6 per cent
Moisture	19.7 per cent

The chilled corn syrup was added to the packed red blood cells, mixed by rotation, and then stored at 4° C.

Seven transfusions of these cells were given after storage for 6 to 22 days.

[5] The human albumin used in this solution was provided through the courtesy of Dr. Edwin Cohn of the Harvard Medical School.

Solutions No. 9, 10, 11, and. 12: Maltose-dextrose solutions

TABLE I

Solution	Name	Disodium citrate	Maltose	Dextrose	NaCl	Vol. of solution	Vol. of packed cells	pH of solution	pH of blood mixture
		per cent	*per cent*	*per cent*	*per cent*		*ml.*		
9	M1	2.0	6.84	0.5	0.85	250	125	4.9	5.7
10	M2	2.0	6.84	0.5	0	250	125	5.0	5.7
11	M3	0.5	6.84	0.75	0	250	125	5.8	5.8
12	M4	0	6.84	0.75	0.43	250	125	6.5	6.7

These chilled solutions were added to packed red blood cells in proportions noted. The cells for solutions No. 9 and 10 were obtained from blood drawn into trisodium citrate solution (solution No. 1). The cells for solutions No. 11 and 12 were drawn into acid citrate dextrose (solution No. 5).

Five transfusions of cells stored 7 to 14 days in these solutions were given.

Solution No. 13: RPA solution [6]

	grams per l.
$MgCl_2$	.095
$CaCl_2$	.056
KCl	.410
NaCl	5.825
Na_2HPO_4	.301
$NaHCO_3$*	2.35
Glucose	2.50
Difco proteose peptone	1.50
Stearns A A**	0.50
Glycerol	0.25
Sodium acetate	0.15
	micrograms per l.
Adenine sulfate	250
Guanine–HCl	250
Thymine	125
Xanthine	250
Uracil	250
Ascorbic acid	5,000
Biotin	16
Choline	500
Cocarboxylase	400
Nicotinic	1,000
Nicotinamide	1,000
d–Ca Pantothenate	500
Pyridoxine	500
Ribose	500
Riboflavin	500
Thiamine	1,000

* Added as Na_2CO_3 and converted to bicarbonate by passing CO_2 gas through the solution.

** Fortified with glycine and histidine.

P-amino benzoic acid is added to conc. 10 gamma per cent.

Medium (complete) is filtered instead of autoclaved.

The solution was added to the red blood cell mass procured from defibrinated blood in amounts sufficient to produce hematocrit values of 27, 37 and 40 per cent. The cells and solution were mixed and the pH adjusted to 7.0 by equilibration with CO_2. The mixture was stored at 4° C.

Solution No. 14: ACD solution 2 per cent

This solution is identical with solution No. 5, but the method of handling the cell-solution mixture differs markedly from that followed with solution No. 5. Four hundred thirty ml. of donor blood were drawn into the chilled solution. The mixture was stored at 4° C. for 24 hours. It was then centrifuged at 2,500 r.p.m. for 1 hour and the supernatant plasma removed aseptically by aspiration. The packed cells were then stored at 4° C. without addition of further diluent until they were transfused. The pH of the cell mass was 6.8. A small amount (equal to 10 per cent of the volume of cells) of 0.85 per cent sodium chloride solution at a temperature of 4° C. was added to the cell mass at the time of transfusion.

Five such transfusions were given of cells stored 11 to 24 days.

Solution No. 15: Acid citrate-dextrose solution 5 per cent

Disodium citrate	(5 per cent)	of this solution	—25 ml.
Dextrose (anhydrous)	(6 per cent)		
Whole blood			250 ml.
pH			5.0
pH of solution-blood mixture			6.8

The blood was drawn into the chilled solution and stored for 24 hours at 4° C. It was then centrifuged at 2,500 r.p.m. for 1 hour and the supernatant plasma removed aseptically by aspiration. The packed cells were stored at 4° C. until transfused and injected without dilution.

Three transfusions were given of cells stored 7 to 22 days.

Solution No. 16: Acid citrate solution 5 per cent

Disodium citrate (5 per cent)	25 ml.
Whole blood	225 ml.
pH of solution	5.0
pH of solution-blood mixture	6.8

The blood was drawn into the chilled solution, stored at 4° C. for 24 hours, centrifuged and the plasma removed as described above. Then 15 ml. of chilled 10 per cent dextrose solution were added to and mixed with the cell mass by rotation of the container. The mixture was stored at 4° C. until transfused and injections were made without dilution, after storage for 9 to 22 days.

Hematologic studies

The following studies were performed on aliquots of donor blood shortly after it was drawn into the preservative solution and at intervals during the period of stor-

[6] This solution was prepared by Dr. Eric Ball of the Harvard Medical School and was identical with the solution used by Ball and his collaborators for the *in vitro* cultivation of malaria parasites (37).

age; they were also performed on aliquots of the samples removed from the recipients before and after the transfusion:

1. Erythrocyte and leukocyte counts were performed in quadruplicate, using 4 pipettes and 4 counting chambers and the average of the 4 counts was used in calculations.
2. Hematocrit determinations were made with the Wintrobe hematocrit tube and with application of a relative centrifugal force of 1,800 for 1 hour.
3. Whole blood hemoglobin concentration was determined by the Evelyn oxyhemoglobin method (38) on the Evelyn photoelectric colorimeter.
4. Erythrocyte indices were calculated according to the method of Wintrobe (39).
5. The concentration of heme pigments in the plasma of the recipient and in the supernatant plasma of the donor blood was determined according to the hemochromogen method of Flink and Watson (40).
6. The plasma bilirubin concentrations of the recipient were determined according to the method of Evelyn and Malloy (41).
7. The whole blood sugar content of the donor blood was determined by the method of Folin and Malmros (42).
8. The pH of the donor blood was determined with the Beckman glass electrode pH meter.
9. The susceptibility of the donor erythrocytes to hemolysis by hypotonic saline solutions ("osmotic fragility") was determined by the method originally suggested by Dacie and Vaughan (43) as modified by Shen, Ham, and Fleming (44).
10. The morphologic characteristics of the donor erythrocytes were observed in wet unstained preparation and in dry Wright stained preparations.
11. Spectroscopic studies were performed on plasma and serum of donor and recipient bloods with the Zeiss hand spectroscope and the Hartridge reversion spectroscope.

OBSERVATIONS

Figures 1 and 2 illustrate the post-transfusion survival of radioactively labeled erythrocytes stored in tridosium citrate (solution No. 1) and in Denstedt's solution (solution No. 2). Following transfusion the non-viable donor cells are destroyed and removed from the recipient's circulation. The number of cells removed and the rapidity with which they are removed increase with increase in the duration of storage.

The majority of the non-viable cells are removed during the first hour or 2 following transfusion and the survival curve then continues to fall more gradually during the next 18 to 20 hours. At the end of 24 hours the minimum post-transfusion survival usually is reached. It is this minimum survival which we have used as the criterion of the effectiveness of the blood preservative, and it is this value which has been used in constructing the curves in Figure 4. After 24 hours newly formed erythrocytes containing reutilized radioactive iron may be liberated in the recipient's circulation more rapidly than the labeled donor cells are removed and consequently the concentration of radioactive iron per unit volume of red cells increases.

Trisodium citrate obviously is a very poor blood preservative (Figure 1). The erythrocytes of stored citrated blood degenerated at a rate of 7 per cent per day and after storage for 7 days only 50 per cent of the transfused cells survived 24 hours (Figures 1 and 4). If we accept as an arbitrary minimum requirement for a satisfactory transfusion the survival of 70 per cent of the transfused cells for at least 24 hours following transfusion, citrated blood can no longer be considered satisfactory for transfusion if stored for longer than 5 days.

Denstedt's solution, in marked contrast to trisodium citrate, is an excellent blood preservative (Figures 2 and 4). Blood stored in this solution for 24 days showed satisfactory post-transfusion survival, and the rate of degeneration of red cells during storage was only approximately 1.4 per cent per day. There was 1 serious drawback to Denstedt's solution, however. The necessity for separately autoclaving the citrate, dextrose, and phosphate solutions and then mixing them with sterile technique made its preparation exceedingly laborious and not very satisfactory for routine use. Since the acid citrate-dextrose solution (solution No. 5) was found to have almost identical preservative powers and was much simpler to prepare, Denstedt's solution does not justify the effort required to prepare it.

The 2 Alsever's solutions studied differed only in their citric acid content and initial pH, and were almost identical in their preservative powers (Figure 3). They were not good preservative solutions, since after storage for only 7 days, less than 70 per cent of the transfused cells survived 24 hours, and the rate of degeneration of the erythrocytes during storage amounted to approximately 3 per cent per day.

Another serious disadvantage of Alsever's solution is the large volume of diluent (500 ml.) required for the preservation of 500 ml. of blood. The addition of this large volume of fluid serves

no useful purpose and is actually contraindicated in many blood transfusions.

The most satisfactory blood preservatives which we studied were the acid citrate-dextrose solutions, either the solution originally described by Loutit and Mollison (14) (solution No. 5) or modifications of this fluid (solutions No. 6, 14, 15, and 16). The acid citrate-dextrose solution of Loutit and Mollison (for convenience called "ACD" throughout the remainder of this paper) was an excellent blood preservative—maintaining satisfactory transfusion properties in blood stored for as long as 23 days (Figure 4) and retarding the rate of degeneration of erythrocytes during storage to only 1.4 per cent per day. In addition to its excellence as a preservative ACD was extremely simple to prepare. The citrate and dextrose solutions were mixed prior to sterilization, and were autoclaved together without caramelization.

The volume of ACD (120 ml.) was not large enough to dilute the blood to any serious degree, but it was believed by the Subcommittee on Blood Substitutes of the National Research Council that a smaller volume preservative might be desirable, since it might make more simple the preparation of desiccated plasma. Therefore, solution No. 6 was devised. Its volume was only 75 ml. but it provided the same amount of disodium citrate per unit volume of blood as solution No. 5; it reduced significantly the amount of dextrose. The preservative properties of this solution were indistinguishable from those of solution No. 5 (Table II).

TABLE II

Survival of erythrocytes of stored whole blood

Preserving solution	Per cent r.b.c. surviving in the recipient's circulation 24° after transfusion of blood stored			
	7 days	14 days	21 days	28 days
1. Trisodium citrate	50	3	0	
2. Denstedt's solution	92	90	73	60
3. Alsever's No. I	67	60	34	13
4. Alsever's No. II	68	60		
5. Acid citrate-dextrose (Loutit and Mollison) ("ACD")	97	92	71	60
6. Acid citrate-dextrose reduced volume	95	92	75	64

Similar observations were obtained with a solution of even smaller volume (50 ml.) (solution No. 15), which was used for preservation of erythrocytes separated from plasma. The post-transfusion survival of the separated cells taken into this solution was almost identical with that of whole blood in 120 ml. volume ACD (solution No. 5) or in the 75 ml. of modified ACD (solu-

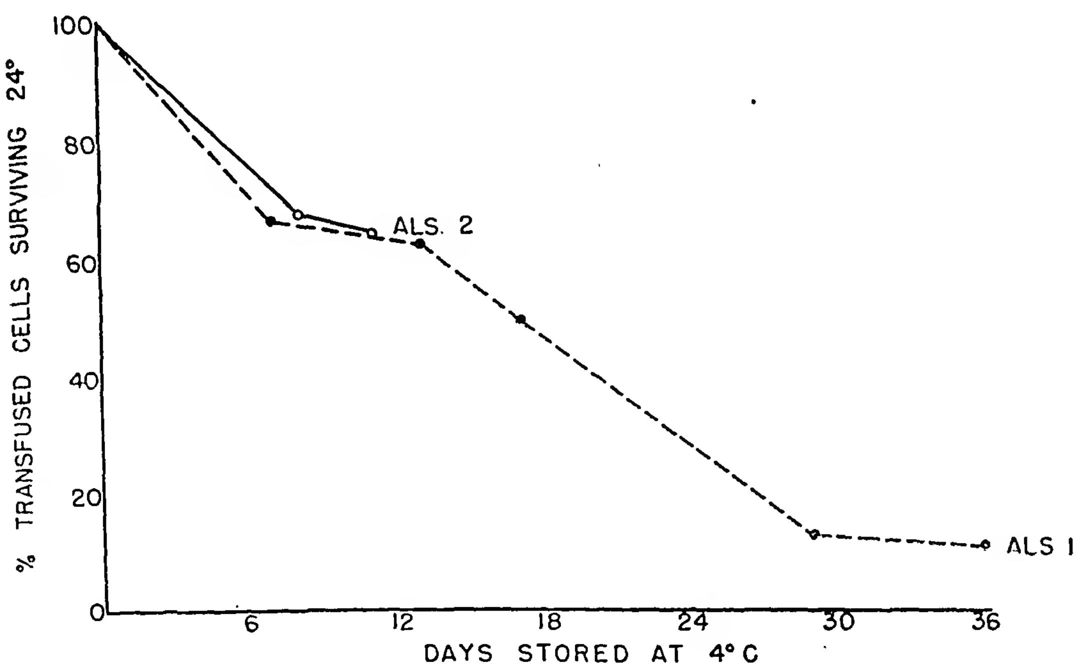

FIG. 3. COMPARISON OF THE EFFECT OF STORAGE OF WHOLE BLOOD IN ALSEVER'S SOLUTIONS NO. 1 AND 2 ON POST-TRANSFUSION SURVIVAL

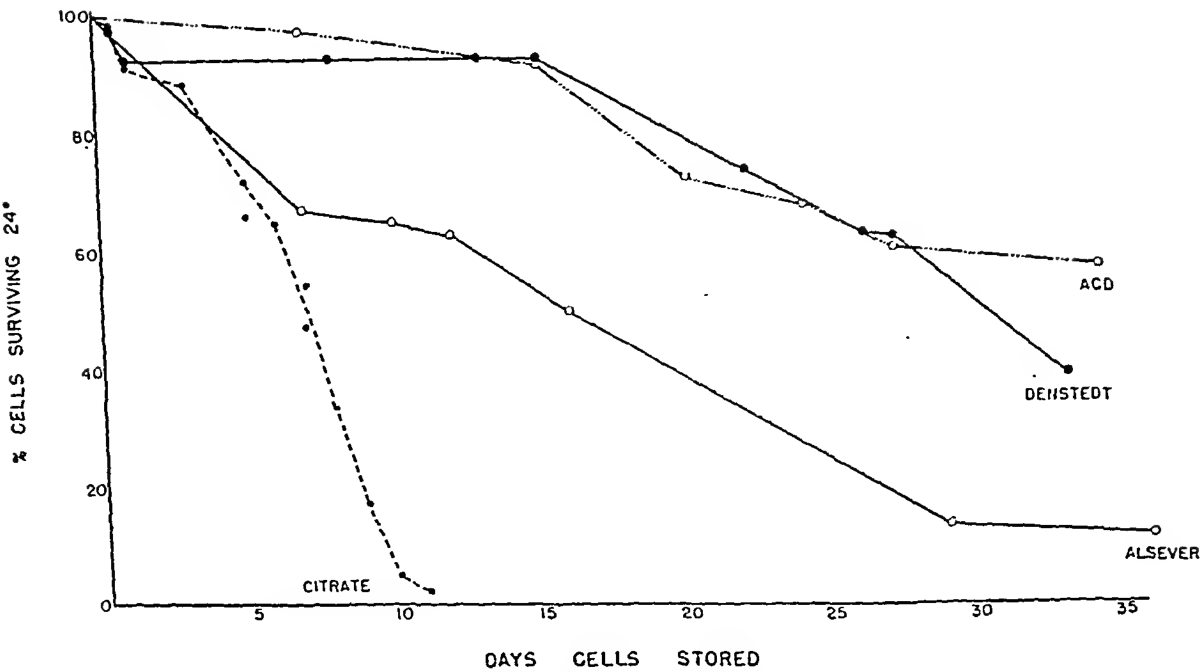

Fig. 4. Comparison of the Survival of the Erythrocytes of Whole Blood Stored at 4° C. in the Acid Citrate-Dextrose Solution of Loutit and Mollison (solution No. 5, "ACD"), Denstedt's Solution (solution No. 2), Alsever's (solution No. 3), and Trisodium Citrate Solution (solution No. 1)

tion No. 6). The volume of the preservative within these limits had little influence on erythrocyte preservation.

Preservation of erythrocytes separated from plasma

Since normal blood contains various hemolytic systems it was believed that erythrocytes might survive better *in vitro* if they could be removed from their plasma and resuspended in some other fluid. Furthermore, if some method of preserving cells separated from plasma could be devised it would salvage tremendous quantities of red blood cells which are discarded in the process of preparing blood plasma and plasma fractions. Such studies were successful in demonstrating a simple yet effective method of preserving red blood cells after separation from plasma.

The studies of Furchgott and Ponder (45) indicated that crystalbumin prevented the development of certain morphologic abnormalities in erythrocytes placed under abnormal conditions *in vitro*. With the hope that human albumin might exert a similar beneficial effect on stored human red cells, we resuspended erythrocytes separated from whole blood in solution No. 7. After storage for 22 days 70 per cent of the transfused cells survived 24 hours (Figure 5), which is good preservation but no better than in several far simpler and less expensive solutions.

This proved to be the case with corn syrup (solution No. 8) which was investigated at the request of the Subcommittee on Blood Substitutes. It maintained satisfactory transfusion properties in resuspended erythrocytes for only 10 days (Figure 5).

In vitro studies indicated that non-diffusible disaccharides might be effective as erythrocyte preservatives; so the maltose-dextrose solutions (solutions No. 9, 10, 11 and 12) were devised to test this possibility. Only solution No. 12 was of any value (Table III).

Ball and his collaborators (37) showed that the malarial parasite could be satisfactorily cultivated in erythrocytes suspended in the complex solution described as "RPA" (solution No. 13). It seemed possible that one of the reasons this solution supported the growth of malarial parasites was because it maintained erythrocyte viability. Therefore, we investigated the preservation of erythrocytes separated from their plasma, resuspended, and stored in this fluid.

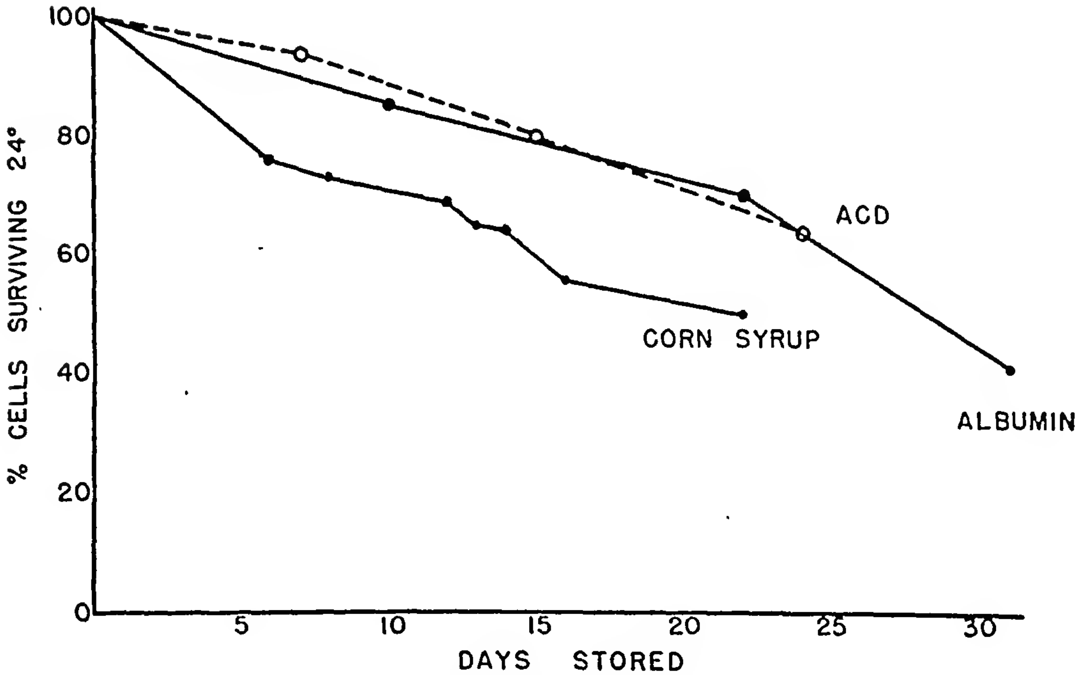

FIG. 5. COMPARISON OF THE POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES SEPARATED FROM PLASMA

ACD represents cells separated from blood drawn into ACD and stored without addition of diluent (solution No. 14). The "albumin" and "corn syrup" cells were resuspended in solutions No. 7 and 8.

The post-transfusion survival of these cells was quite good (Table III), but, as in the case of the albumin solution, corn syrup, and maltose-dextrose solutions, it was not so good as the survival of cells stored in simpler and less expensive preservatives.

The excellence and simplicity of the ACD solutions (solutions No. 5 and 6) as whole blood preservatives and the fact that plasma was not essential to the preservation of erythrocytes (solutions No. 7, 8 and 12) suggested that red blood cells might survive if they were separated from blood drawn into ACD and stored without resuspension (solution No. 14). The survival of cells preserved in this fashion was excellent (Figure 5). A smaller volume ACD (solution No. 15) proved just as effective as solution No. 14 (Figure 6).

TABLE III

The survival of erythrocytes separated from plasma

Resuspending solution or technique	Per cent r.b.c. surviving in the recipient's circulation 24° after transfusion of blood stored		
	7 days	14 days	21 days
7. Human albumin	90	80	72
8. Corn syrup	74	64	51
9. Maltose No. M1	43	25	
10. Maltose No. M2	0		
11. Maltose No. M3		20	
12. Maltose No. M4	81	72	
13. RPA solution	90	80	63
14. ACD (2 per cent solution) packed cells not resuspended	94	80	70
15. ACD (5 per cent solution) packed cells not resuspended	92	78	70
16. AC (5 per cent solution) + dextrose added to packed cells after 24°	97	90	75

In the recovery of plasma it may be desirable in some instances not to add dextrose directly to the preservative solution until the plasma has been removed. To investigate the possibility of preserving the erythrocytes of such blood we carried out the procedure outlined in solution No. 16. Blood was drawn into 5 per cent disodium citrate solution, the plasma was removed the next day, and dextrose solution was added to the cell mass. As summarized in Table III, the survival of these erythrocytes was as good, if not slightly better than the survival of cells drawn directly into ACD.

Influence of storage temperature on post-transfusion erythrocyte survival

Although Rous and Turner (3) in their original observations clearly demonstrated the importance

of refrigeration in the preservation of stored blood, a recent publication questioned its necessity and claimed that certain preservatives were capable of maintaining erythrocyte viability at room temperature (46). Large amounts of unrefrigerated whole blood were being sent to the European Theatre of Operations in 1944, and since poor preservation of this blood might render it potentially dangerous to recipients the question of refrigeration was reinvestigated.

In the first series of observations, donor blood was drawn directly into Alsever's solution (solution No. 4) at room temperature (25° C.) and allowed to remain at room temperature until transfused. As illustrated in Figure 7 the post-transfusion survival of these erythrocytes was extremely poor. After 2 days' storage only 65 per cent of erythrocytes survived 24 hours in the recipient's circulation, and after 4 days, only 25 per cent of the cells survived 24 hours.

When blood was taken into chilled ACD, stored at 4° C. for 3.5 days, and then at room temperature (25° C.) until transfusion, erythrocyte survival was extremely poor, and after 9 days' storage, none of the labeled cells survived (Figure 7).

The adverse effect of warm temperature was not overcome by returning the blood to refrigeration after it had been exposed for relatively brief periods of time to room temperature. Blood drawn into chilled Alsever's solution, refrigerated at 4° C. for 2 days, exposed to room temperature (25° C.) for 24 hours, and then returned to 4° C., showed considerable decrease in post-transfusion survival (Figure 7).

These studies emphasize the necessity for constant refrigeration of whole blood and of erythrocytes during storage and reconfirm the original observations of Rous and Turner.

Practical application of acid citrate-dextrose solutions

The acid citrate-dextrose solution of Loutit and Mollison (solution No. 5) and the small volume ACD (solution No. 15) were given extensive practical trials in the blood bank of the Massachusetts Memorial Hospitals. From September, 1944 until January, 1946 the ACD of Loutit and Mollison (solution No. 5) was used for all routine bleedings in the blood bank. During this period 2,576 bloods were taken into this solution, 1,926 transfusions were given, and 217 pints of pooled, frozen plasma were prepared.

The maximum period of storage of this blood was 21 days although most bloods were transfused within the first 10 days after they were drawn. The therapeutic responses to these transfusions

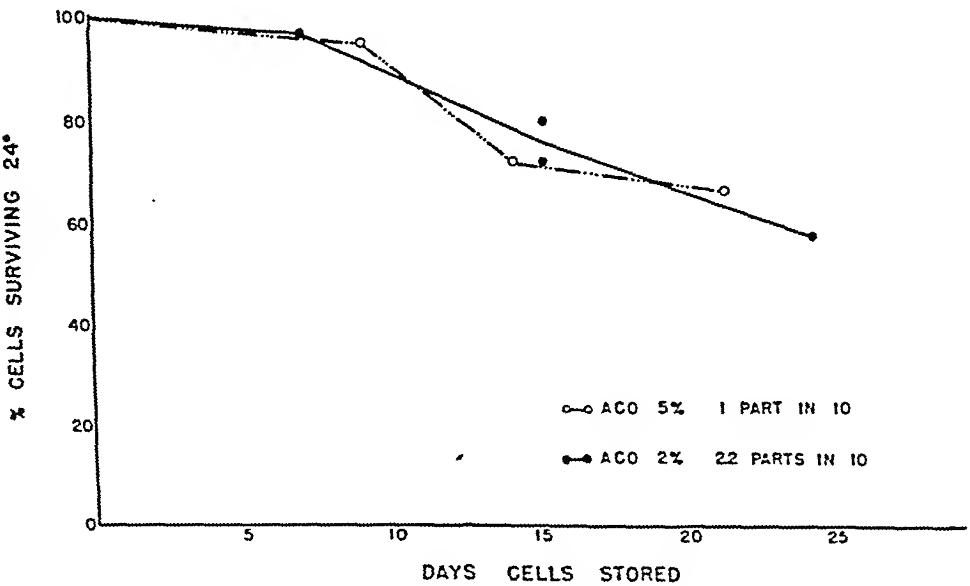

Fig. 6. The Effect of Variation in the Volume of Preservative on the Post-transfusion Survival of Erythrocytes Separated from Plasma

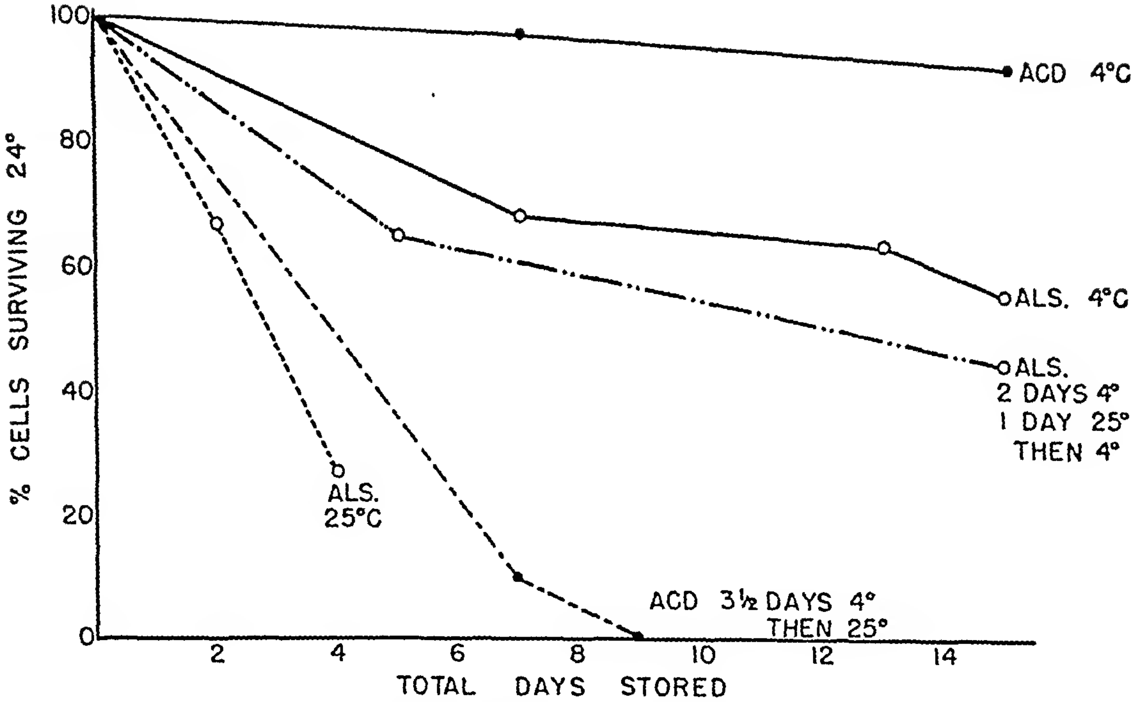

FIG. 7. THE EFFECT OF VARIATION IN STORAGE TEMPERATURE ON THE POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES OF WHOLE BLOOD

were completely satisfactory, and the incidence of reactions was not higher than that usually prevailing in this blood bank.

The concentration of the citrate ion in the supernatant plasma of the ACD blood mixture is approximately 24 millimolars—a value approximately double the concentration needed to prevent coagulation (47). In spite of this fact, however, there is a tendency to clot formation in blood drawn into this solution unless the blood and anticoagulant are thoroughly mixed during the process of venesection. If this precaution is observed, the incidence of clot formation is no higher than with other preservative fluids, and in our experience has presented no serious difficulty.

From January, 1946 to February, 1947 the 50 ml. of 5 per cent ACD solution (solution No. 15) was used for routine bleedings; 2,522 bloods were drawn into this solution, 2,002 transfusions were dispensed, and 181 pints of frozen plasma were prepared. This preservative was in every respect as satisfactory as the larger volume ACD preservative.

Packed red blood cells separated from their plasma were routinely prepared from bloods drawn into each solution as described for solutions No. 14 and 15. They have proven eminently satisfactory therapeutically, and their use in transfusions for the correction of anemia is steadily increasing.

COMMENT

Loss of viability of stored erythrocytes proceeds at a very constant rate in blood stored in any given preservative, suggesting that the degenerative process may be one of aging and similar to the process normally going on in the body. The steadiness of the rate of senescence is well illustrated in Figures 4, 5, and 7 in which it appears that the decrease in viable erythrocytes is a straight line function of time. It is possible to calculate the approximate rate of senescence per day for blood stored in different preservatives and such rates are tabulated in Table IV. The rate is high in poor preservatives and at room temperature, and low in good preservatives and in the cold. In all preservatives studied it is higher than the 0.83 to 1.0 per cent per day rate of degeneration of erythrocytes in the body.

Following injection into a recipient, most of the non-viable erythrocytes are removed from the circulation very rapidly—usually within an hour or two. A smaller number are removed at a less rapid rate during the next 10 to 20 hours (Figures 1 and 2). The cells which are removed rapidly

TABLE IV

Maximum allowable duration of storage of blood and separated erythrocytes in various preservatives

Preservative: Solution no.	Preservative: Name	Maximum duration storage permitting 70 per cent post-transfusion survival	Approximate rate of degeneration of r.b.c. *in vitro*
		days	*per cent per day*
	Whole blood		
2	Denstedt's	24	1.4
6	Acid citrate-dextrose	23	1.4
5	Acid citrate-dextrose (Loutit and Mollison)	22	1.4
4	Alsever's	7	3.0
1	Trisodium citrate	5	7.0
	Separated cells		
14	R.b.c. from blood in ACD of Loutit and Mollison	20	1.5
16	R.b.c. from blood in 5 per cent acid citrate. Dextrose added later	24	1.3
15	R.b.c. from blood in 5 per cent ACD	20	1.5
7	R.b.c. suspended in albumin solution	22	1.4
13	R.b.c. suspended in RPA fluid	18	1.7
12	Maltose-dextrose	15	2.0
8	Corn syrup	10	3.0

probably are dead prior to injection; those removed at the slower rate probably have been damaged during storage and become non-viable after injection.

The longer a blood has been stored and the more non-viable erythrocytes there are—the more rapid is the rate at which the non-viable cells are removed. This may be accounted for by progressive changes in the stored erythrocytes after they have become non-viable—a "postmortem degeneration," so to speak. If these changes are extensive enough they may result in actual disintegration of the erythrocytes and be reflected by gross hemolysis in the stored blood. As described in a subsequent paper, however, extensive degenerative changes may occur in erythrocytes stored in some preservatives, without gross hemolysis or other evidence of cell disintegration.

Our studies have been directed primarily at a critical evaluation of the effectiveness of certain selected preservatives, rather than at a general survey of the properties of preservative fluids. However, there are certain important features of blood preservatives which have become apparent in our studies, and these will be commented on briefly. The presence of dextrose in a preservative solution is essential for maintenance of erythrocyte viability. Its actual concentration does not appear to be critical within the range of 0.5 to 2.0 per cent. The initial reaction of the preservative-blood mixture is important and should be between pH 6.8 and 7.0. Certain autolytic or destructive processes probably are retarded at this pH. The degree of dilution of the blood with preservative fluid is not of great importance. The survival of erythrocytes stored in a cell mass with a hematocrit of 88 per cent is little different from the survival of cells stored in a whole blood-preservative mixture with a hematocrit of 30 per cent. The presence of plasma in the preserving medium is not essential for good erythrocyte preservation, and there is some evidence that its removal may improve preservation. The importance of constant refrigeration has already been emphasized. The addition of vitamins, amino acids, and other accessory substances is not essential and does not improve erythrocyte preservation.

In adopting the arbitrary value of 70 per cent post-transfusion survival as a minimum requirement for satisfactory transfusion properties, we do not wish to imply that blood providing cells of this viability is as satisfactory as blood in which a greater percentage or all of the cells are viable. Although blood stored for 3 weeks and providing erythrocytes of 70 per cent viability may be "satisfactory" for transfusion under emergency circumstances, it is not so good as fresh blood or blood stored for a shorter period of time. During the war when large quantities of blood had to be shipped literally to the ends of the earth it was occasionally necessary to use blood stored for 3 weeks, and such blood saved the lives of thousands of American troops. In civilian practice and in most hospital blood banks there is no necessity for using blood stored this long.

From a practical standpoint, however, transfusion of blood stored in ACD, or one of the modifications of ACD, for 7 to 10 days may be considered as satisfactory as fresh blood for the relief of anemia.

SUMMARY

(*1*) The effectiveness of 16 solutions and of various techniques in the preservation of whole

blood, and of erythrocytes separated from plasma have been evaluated with the radioactively tagged erythrocyte technique.

(*2*) The most effective preservative and the simplest to prepare was an acid citrate-dextrose solution.

(*3*) Whole blood drawn into this solution maintained satisfactory transfusion properties during a storage period of 21 days.

(*4*) Erythrocytes separated from blood drawn into this acid citrate-dextrose solution and stored without addition of diluent also maintained satisfactory transfusion properties during a storage period of 21 days.

(*5*) This acid citrate-dextrose solution proved to be very satisfactory in routine hospital blood bank use.

(*6*) Studies of the influence of temperature on the viability of stored erythrocytes emphasized the necessity for constant refrigeration of blood during the entire period of storage.

ACKNOWLEDGMENT

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BIBLIOGRAPHY

1. Emerson, C. P., Jr., and Ebert, R. V., A study of shock in battle casualties, measurements of the blood volume changes occurring in response to therapy. Ann. Surg., 1945, 122, 745.
2. Ross, J. F., and Chapin, M. A., Effect of storage of citrated blood on the survival of transfused erythrocytes. J. A. M. A., 1943, 123, 827.
3. Rous, P., and Turner, J. R., The preservation of living red blood cells *in vitro*. J. Exper. Med., 1916, 23, 219.
4. Robertson, O. H., Transfusion with preserved red blood cells. Brit. M. J., 1918, 1, 691.
5. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. I. Various factors influencing hemolysis. J. A. M. A., 1940, 114, 850.
6. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. II. Diffusion of potassium from the erythrocytes during storage. J. A. M. A., 1940, 114, 855.
7. Belakhouski, S. D., Ginzburg, F. G., Palichina, A., Rzechina, S. V., and Farberova, R., Preservation of blood intended for blood transfusion. Sovr. probl. gematal., 1932, 3–4, 16.
8. Yudin, S. S., Transfusion of stored cadaver blood; practical considerations: the first thousand cases. Lancet, 1937, 2, 361.
9. Jorda, F. D., The Barcelona blood transfusion service. Lancet, 1939, 1, 773.
10. Fantus, B., The therapy of Cook County Hospital: blood preservation. J. A. M. A., 1937, 109, 128.
11. Alsever, J. B., and Ainslie, R. B., A new method for the preparation of dilute blood plasma and the operation of a complete transfusion service. New York State J. Med., 1941, 41, 126.
12. Mollison, P. L., and Young, I. M., On the survival of the transfused erythrocytes of stored blood. Quart. J. Exper. Physiol., 1941, 30, 313.
13. Mollison, P. L., and Young, I. M., *In vivo* survival in the human subject of transfused erythrocytes after storage in various preservative solutions. Quart. J. Exper. Physiol., 1942, 31, 359.
14. Loutit, J. F., and Mollison, P. L., Advantages of a disodium citrate-glucose mixture as a blood preservative. Brit. M. J., 1943, 2, 744.
15. Loutit, J. F., Mollison, P. L., and Young, I. M., Citric acid-sodium citrate-glucose mixtures for blood storage. Quart. J. Exper. Physiol., 1943, 32, 183.
16. Loutit, J. F., Factors influencing the preservation of stored red cells. J. Path. and Bact., 1945, 57, 325.
17. Denstedt, O. F., Osborne, D. E., Stansfield, H., and Rochlin, I., The survival of preserved erythrocytes after transfusion. Canad. M. A. J., 1943, 48, 477.
18. Denstedt, O. F., Osborne, D. E., Stansfield, H., and Rochlin, I., The survival of preserved red cells after transfusion. Anesthesiology, 1944, 5, 237.
19. Ashby, W., The determination of length of life of transfused blood corpuscles in man. J. Exper. Med., 1919, 29, 267.
20. Wiener, A. S., Longevity of erythrocytes. J. A. M. A., 1934, 102, 1779.
21. Mollison, P. L., and Young, I. M., Failure of *in vitro* tests as a guide to the value of stored blood. Brit. M. J., 1941, 2, 797.
22. Maizels, M., Phosphate, base and hemolysis in stored blood. Quart. J. Exper. Physiol., 1943, 32, 143.
23. Hahn, P. F., Bale, W. F., Ross, J. F., Hettig, R. A., and Whipple, G. H., Radio-iron in plasma does not exchange with hemoglobin iron in red cells. Science, 1940, 92, 131.
24. Ross, J. F., and Chapin, M. A., The electrolytic separation of radioactive iron from the blood. Rev. Scient. Instruments, 1942, 13, 77.
25. Peacock, W. C., Evans, R. D., Irvine, J. W., Jr., Good, W. M,. Kip, A. F., Weiss, S., and Gibson, J. G., The use of two radioactive isotopes of iron in tracer studies of erythrocytes. J. Clin. Invest., 1946, 25, 605.
26. Ebert, R. V., and Emerson, C. P., Jr., A clinical study of transfusion reactions: the hemolytic effect of group-O blood and pooled plasma containing incompatible isoagglutinins. J. Clin. Invest. 1946, 25, 627.

27. Heller, V. G., and Paul, H., Changes in cell volume produced by varying concentrations of different anticoagulants. J. Lab. & Clin. Med., 1934, 19, 777.
28. Chapin, M. A., and Ross, J. F., The determination of the true cell volume by dye dilution, by protein dilution, and with radioactive iron. The error of the centrifuge hematocrit. Am. J. Physiol., 1942, 137, 447.
29. Hahn, P. F., Ross, J. F., Bale, W. F., Balfour, W. M., and Whipple, G. H., Red cell and plasma volumes (circulating and total) as determined by radio iron and by dye. J. Exper. Med., 1942, 75, 221.
30. Gibson, J. G., 2nd, Weiss, S., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., Good, W. M., and Kip, A. F., The measurement of the circulating red cell volume by means of two radioactive isotopes of iron. J. Clin. Invest., 1946, 25, 616.
31. Ross, J. F., Chapin, M. A., Sammons, M. E., and Paegel, B. L., The total red blood cell volume of normal human subjects determined with erythrocytes labeled with radioactive iron. Blood. (In press.)
32. Gibson, J. G., 2nd, and Evelyn, K. A., Clinical studies of the blood volume. IV. Adaptation of the method to the photoelectric microcolorimeter. J. Clin. Invest., 1938, 17, 153.
33. Price, P. B., and Longmire, W. P., The use of T-1824 in plasma volume determinations. Bull. Johns Hopkins Hosp., 1942, 71, 51.
34. Noble, R. P., and Gregersen, M. I., Blood volume in clinical shock. I. Mixing time and disappearance rate of T-1824 in normal subjects and in patients in shock; determination of plasma volume in man from 10-minute sample. J. Clin. Invest., 1946, 25, 158.
35. Ross, J. F., Unpublished observations.
36. Thalhimer, W., and Taylor, E. S., Transfusion of centrifuged human type O cells resuspended and stored in 10 per cent corn syrup. J. A. M. A., 1945, 127, 1096.
37. Ball, E. G., Anfinsen, C. B., Geiman, Q. M., McKee, R. W., and Ormsbee, R. A., *In vitro* growth and multiplication of the malarial parasite, *Plasmodium knowlesi*. Science, 1945, 101, 542.
38. Evelyn, K. A., A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., 1936, 115, 63.
39. Wintrobe, M. M., Size and hemoglobin content of erythrocytes; methods of determination and clinical application. J. Lab. & Clin. Med., 1932, 17, 899.
40. Flink, E. B., and Watson, C. J., Method for quantitative determination of hemoglobin and related heme pigments in feces, urine, and blood plasma. J. Biol. Chem., 1942, 146, 171.
41. Malloy H. T., and Evelyn, K. A., The determination of bilirubin with the photoelectric colorimeter. J. Biol. Chem., 1937, 119, 481.
42. Folin, O., and Malmros, H., Improved form of Folin's micro method for blood sugar determination. J. Biol. Chem., 1929, 83, 115.
43. Dacie, J. V., and Vaughan, J. M., Fragility of red blood cells: its measurement and significance. J. Path. & Bact., 1938, 46, 341.
44. Shen, S. C., Ham, T. H., and Fleming, E. M., Studies on the destruction of red blood cells. III. Mechanisms and complications of hemoglobinuria in patients with thermal burns. Spherocytosis and increased osmotic fragility of red blood cells. New England J. Med., 1943, 229, 701.
45. Furchgott, R. F., and Ponder, E., Disk-sphere transformation in mammalian red cells. II. The nature of the anti-sphering factor. J. Exper. Biol., 1940, 17, 117.
46. Kendrick, D. B., Jr., Elliott, J., Reichel, J., Jr., and Vaubel, E. K., Supply of preserved blood to European theatre of operations, a preliminary report. Bull. U. S. Army M. Dept. (No. 84), 1945, 3, 66.
47. Maizels, M., Observations on stored blood, report B. T. 30, to the Medical Research Council, Blood Transfusion Research Committee, January, 1943.

THE MEASUREMENT OF POST-TRANSFUSION SURVIVAL OF PRESERVED STORED HUMAN ERYTHROCYTES BY MEANS OF TWO ISOTOPES OF RADIO-ACTIVE IRON [1]

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The evaluation of the efficacy of whole blood transfusion in augmenting total red cell volume has always been of interest to physicians called upon to care for patients with hemorrhage, burns, blood dyscrasias, or traumatic shock. The establishment of blood banks, resulting in an increasing use of stored blood, has focused more attention on the problem. Military requirements have created a demand for the preservation of whole blood over far longer periods than are required for civilian purposes. The urgent need for better preservative solutions and for the selection of the best conditions for overseas air transport of whole blood for the Armed Forces made it imperative that an accurate method of measuring the post-transfusion survival of stored human erythrocytes be available.

The morphological and chemical changes that take place in red cells during storage have been studied by several laboratory methods. The rate of spontaneous hemolysis, changes in cell dimension, changes in the permeability of the cell membrane, changes in osmotic resistance to hypotonic solutions of NaCl, rate of diffusion of potassium, and disturbances in carbohydrate metabolism have all been proposed as *in vitro* tests for the evaluation of the ability of stored red cells to survive after transfusion.

Each of these tests assays only changes in one functional characteristic of the erythrocyte and it is for this reason that the opinion has been expressed that *in vitro* tests fail as a guide to the viability of stored blood (1). When several of these techniques have been applied in conjunction, they have served a useful purpose in screening suggested preservative solutions for further evaluation. But, until the changes demonstrated by *in vitro* studies are proved to reflect truly the degree of red cell deterioration, the final evaluation of any preservative must be based on the results of *in vivo* post-transfusion survival studies.

The simplest *in vivo* method is the measurement of the recipient's red cell count, hemoglobin, or hematocrit before and after transfusion. In uncomplicated anemia or blood loss this procedure does give some idea of the improvement in red cell volume, but in hemolytic anemias or in the presence of continuing hemorrhage the interpretation of data is difficult or impossible. It has been our experience that the hematocrit and hemoglobin level of venous blood, as well as the red cell count from finger blood, vary greatly in random samples from a given individual, depending upon the degree of venous stasis incident to venipuncture, temperature of extremities, and the general circulatory state of the subject. This variation may be as great as the net increase in hematocrit, hemoglobin, or red count expected to result from a single 500-ml. transfusion.

The increase in circulating red cell volume resulting from one or more transfusions may be measured by the dye-plasma volume hematocrit technic with a fair degree of accuracy. A transfusion of 500 ml. of whole blood contains about 200 ml. of erythrocytes. This quantity is about 10 per cent of the normal human circulating red cell volume (2). In hemorrhage or blood loss the percentage of transfused to recipient circulating red cells varies directly with the degree of cell volume

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts Institute of Technology, in collaboration with the Peter Bent Brigham Hospital, and the Massachusetts General Hospital.

deficit. A single transfusion will rarely represent more than 30 per cent of the pre-transfusion erythrocyte volume.

Plasma volume can be measured by the dye method within ± 5 per cent. An additional error is introduced by unavoidable variations in the hematocrit determination on venous blood samples, upon which the red cell volume is calculated. Circulating red cell volume measured by the dye-plasma volume hematocrit technic is greater than the true volume (3, 4, 5), because the hematocrit of capillary blood is less than that of blood in large vessels (6, 7). This error may be minimized by correcting the calculated cell volume by the factor 0.85. Hence, the intrinsic error of the technic, which is about ± 5 per cent, may be the equivalent of a large fraction of the cells transfused. It is desirable to follow the fate of stored cells at frequent intervals after transfusion, but the dye method gives only data for a single period, and repeated blood volume determinations at short intervals are not always practicable.

The occurrence of hemoglobinuria or hemoglobinemia following a compatible transfusion is, of course, evidence of massive destruction of donor or recipient cells. Hemolysis produces a rise in recipient serum bilirubin levels. Strumia has shown a progressive hyperbilirubinemia with increasing age of stored blood (8). Gilligan and Altschule (9) found large variations in the net rise in plasma hemoglobin levels following the intravenous administration of hemoglobin solutions, indicating wide individual differences in the ability to handle pigments derived from blood. Bilirubin data are misleading in patients with liver disease or hemolytic tendencies. The measurement does not permit quantitative evaluation of cell destruction.

The agglutination method of following the fate of transfused cells was introduced by Ashby in 1919 (10, 11). Whole blood from a group O donor is given to a group A recipient. The transfused O cells are, of course, of all different ages and represent mixed-age cells. Samples of recipient blood are taken and the A cells agglutinated with anti-A typing serum and thrown down by centrifugation. The unagglutinated O cells remaining in suspension are then counted with standard blood counting pipettes and chambers. The count in a sample drawn at the completion of the transfusion or shortly thereafter is usually taken as the quantity of O cells in circulation resulting from complete retention of all the transfused cells. Counts upon subsequent samples are referred to this "100 per cent value" for calculation of the percentage of surviving O cells. The method has been widely used in the study of red cell preservation by Mollison (12), Maizels (13), Denstedt (14), and more recently by Thalhimer (15).

Scrutiny of the results obtained by these workers shows that the non-agglutinable counts on samples taken only a few days apart may vary by as much as 20 per cent (14, 15), even when the greatest possible care is taken. This suggests a large intrinsic probable error in the method.

All workers report that a certain proportion of the recipient A cells are not agglutinated by anti-A sera, even though of very high titre. In practice this non-agglutinable cell count is determined prior to transfusion and subtracted from subsequent counts. The assumption is that this non-agglutinable portion of recipient cells remains a constant throughout the observation period, which in some instances has been as long as 130 days. Such an assumption may or may not be valid.

It is assumed that a complete separation of the unagglutinated O from the agglutinated A cells is effected by the addition of serum and subsequent shaking and centrifugation. It is, however, possible that numbers of the O cells may become enmeshed in the large A agglutinates and carried down therewith in centrifuging.

It has been suggested (14) that in instances in which there is an abrupt rise in cell count from the previously prevailing slope of cell disappearance, some of the transfused cells may have been temporarily withdrawn from circulation (in the spleen or liver), and later have re-entered the circulation. This hardly seems likely, since it is improbable that there are any great "depots" of red cells in the body but rather that in the normal state practically all the erythrocytes are in active circulation at all times (7).

The agglutination technic permits one to follow the presence of transfused O cells throughout their entire life span in the recipient, information that cannot be obtained by the radioactive technic.

A method of measuring post-transfusion survival of human erythrocytes by means of radioactive isotopes of iron was first described by Ross and Chapin in 1943 (16). The modifications de-

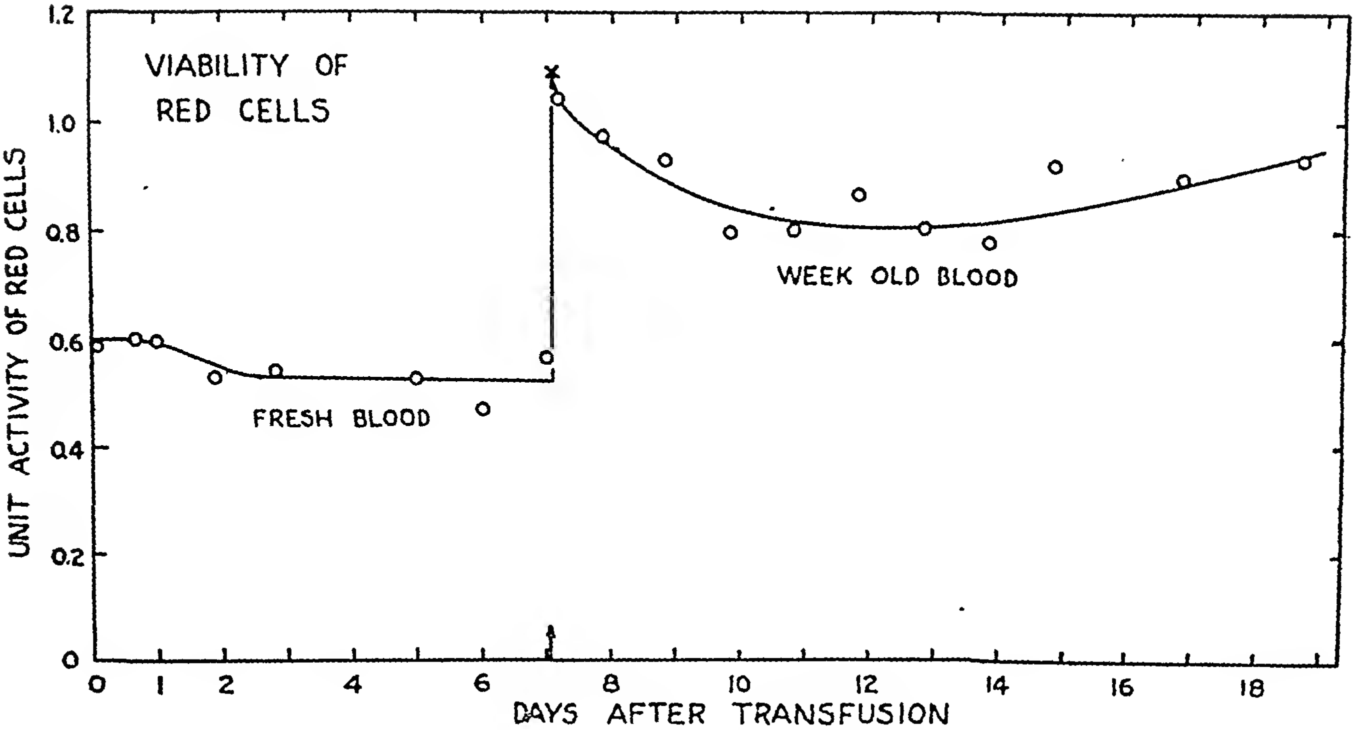

FIG. 1. VIABILITY OF HUMAN ERYTHROCYTES STORED AS WHOLE BLOOD IN 4 PER CENT SODIUM CITRATE

Five hundred ml. of whole blood from a radioactive donor was taken in 4 per cent sodium citrate and divided into 2 equal parts. One aliquot was transfused on the day drawn, and the other given to the same recipient after refrigerated storage for 7 days. The Ua of the recipient's red cells following the first transfusion indicates good retention of the tagged cells for 1 day and a slight loss on the second day. After the second transfusion the recipient Ua rose, but not to twice the level resulting from the first transfusion. Since both aliquots contained the same amount of radioactivity, some of the tagged cells from the second transfusion disappeared from the circulation during the administration of the blood. Only about ½ of the 7-day old cells were viable as shown by the fall in recipient Ua through the twelfth day. The subsequent rise in Ua is due to re-utilization of iron from non-viable cells in hemoglobin synthesis.

scribed herein were developed from our studies in measuring circulating red cell volume in shock. The physical principles involved, the characteristics of the iron isotopes used, the apparatus for radioactivity detection, as well as the experimental techniques of using tagged human erythrocytes for cell volume determination have been described previously (17, 18). For the purposes of this study a brief resumé will suffice.

When small amounts of radioactive ferric ammonium citrate (0.5 to 1.5 mgm.) are given intravenously, the salt is rapidly removed from plasma and deposited in body iron stores (18). In the process of erythrocyte formation in the marrow, some of the iron atoms that become incorporated in the hemoglobin molecule of developing cells are radioactive. Since hemoglobin does not escape from the healthy erythrocyte, it follows that as long as the tagged red cells remain intact, their presence may be detected in the blood stream. If these cells are destroyed, the released radioactive iron returns from the plasma to the iron stores, and eventually is re-utilized and resynthetized into hemoglobin, again appearing in new red cells in the blood stream. Thus the immediate retention (24 to 48 hours) of stored tagged cells can be determined, and the eventual utilization of the iron from those cells that did not survive can subsequently be measured.

Data obtained in the first experiment of this type which we carried out are shown in Figure 1. Following a transfusion of fresh radioactive whole blood (drawn in 4 per cent sodium citrate), the recipient's cell Unit Activity (Ua) [2] remained constant for 24 hours, followed by a slight fall from

[2] Defined as counts per minute (cpm) per ml. of red cells referred to cpm of a radioactive iron standard measured at the same time, and therefore independent of the rate of radioactive decay of the active isotope and counter variability.

the initial level, indicating that about 90 per cent of the transfused cells were retained. On the eighth day a transfusion of week-old cells from the same blood donor, and equal in amount to the first, was given. There was a rise Ua to almost, but not quite, twice that resulting from the first infusion. During the next 5 days Ua progressively dropped, leveling off at a value corresponding to a retention of about 50 per cent of the cells given in the second transfusion. Thereafter Ua rose to about 75 per cent of the original level. Since the only source of radioactive iron in the subject was the transfused red cells, it follows that about ½ of the iron released from destroyed cells was re-utilized by the end of the observation period.

This experiment brings out a fact of importance. The level to which Ua rose immediately following the second transfusion was less than twice the level following the first transfusion even though both contained an equal amount of radioactivity. It is, therefore, evident that the initial post-transfusion activity level following the second transfusion did not correspond to 100 per cent retention, but that some of the tagged cells were withdrawn from circulation *while the blood was being administered.* Thus it is essential to supplement this simple procedure with an independent method of determining the radioactivity level corresponding to 100 per cent retention of the transfused tagged red cells.

It is therefore necessary to determine the recipient's circulating cell volume at the time the transfusion is given. In some of our experiments this was determined by the dye-plasma volume hematocrit technic. The calculated cell volume was corrected by the factor 0.85 which we have found to be the average ratio of the values for the true cell volume measured by radio-iron and the cell volume calculated from the dye-plasma-hematocrit (5). Post-transfusion survivals calculated upon these values probably are accurate to within ± 5 per cent.

In many experiments, the recipient's pre-transfusion red cell volume was more accurately measured by a small infusion of fresh group O cells tagged with Fe^{59}. The preserved transfused cells were tagged with Fe^{55}. Both isotopes can be measured in the same blood sample (17).

The transfused quantity of preserved stored cells tagged with Fe^{55} and the radioactivity of those cells is measured. Knowing the recipient's red cell volume the radioactivity level corresponding to 100 per cent retention of the transfused cells is calculated by the equation

$$Ua_T = \frac{C \times UaD}{Vrr_1 + Vrr_2}$$

where

Ua_T is the 100 per cent value of the unit activity Ua.

C is the quantity of cells transfused in ml.

UaD is the unit activity of those cells, and

Vrr_1 is the recipient's pre-transfusion red cell volume in ml. as measured by Fe^{59}.

Vrr_2 is the quantity of stored cells transfused.

The percentage of radioactive cells in the recipient's circulation at any time in terms of the quantity transfused therefore is

$$\frac{UaR \times 100}{UaT},$$

where UaR is the recipient's red cell radioactivity level.

A typical experiment in which recipient cell volume was measured by radio-iron is shown in Figure 2. The subject was a normal 22-year-old male, blood group A, who had been bled 1 day prior to transfusion. On the morning of the experiment plasma volume was determined by the dye method. At the same time, radio-iron red cell volume was determined, the subject receiving 44.8 ml. of cells drawn as whole citrated blood from a group O donor who had been built up with Fe^{59}. Three venous blood samples were drawn at 10-minute intervals after the injection of dye and tagged cells for measurement of plasma dye concentration and red cell radioactivity. Four hours later the subject received 160 ml. of cells tagged with Fe^{55}, drawn as whole blood in Alsever's solution and stored 19 days.

Venous blood samples were then drawn at 20 minutes and approximately 1, 4, and 8 hours after the end of the transfusion of stored blood. Two blood samples were taken on both the first and second day, and 1 sample on the third, fourth, and fifth day after transfusion, and a final sample was taken on the twenty-eighth day. All of these samples were analyzed for both Fe^{59} and Fe^{55}. Data obtained are given in Protocol GR–78 and are plotted in Figure 2.

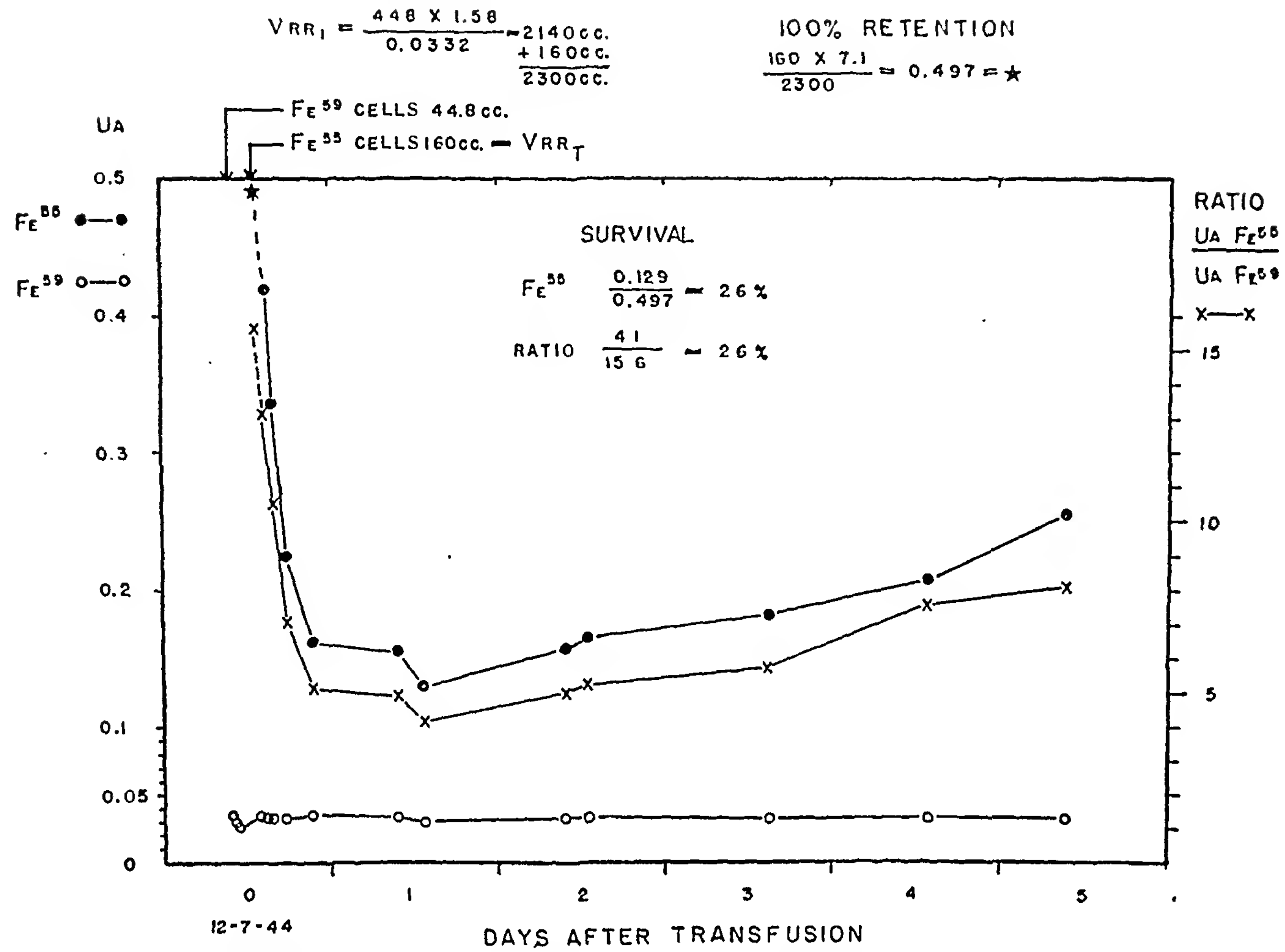

Fig. 2. Method of Measuring Post-transfusion Survival of Stored Human Erythrocytes by means of 2 Isotopes of Radioactive Iron

The recipient's pre-transfusion circulating red cell volume was determined by giving 44.8 ml. of freshly drawn group O cells tagged with Fe^{59} and found to be 2,140 ml. Then 160 ml. of group O cells tagged with Fe^{55} and stored 19 days were given. The resultant expected red cell volume was 2,300 ml. The theoretical recipient red cell Ua corresponding to 100 per cent retention of the Fe^{55} cells was 0.497. The lowest recipient Fe^{55} Ua value was 0.129, and survival was computed at 26 per cent.

Recipient's pre-transfusion red cell volume computed from the quantity of Fe^{59} cells given, the Ua thereof, and the average Fe^{59} Ua value of the first 4 recipient's blood samples, is

$$\frac{44.8 \times \text{Ua Fe}^{59}\ (1.58)}{0.0332} = 2{,}140 \text{ ml.}$$

This agrees well with the calculated corrected dye-plasma-hematocrit red cell volume of 2,110 ml.

The total quantity of recipient circulating red cells is equal to the red cell volume plus the amount of cells transfused, or 2,140 ml. + 160 ml. =2,300 ml.

The recipient's 100 per cent retention value Fe^{55} Ua then becomes

$$\frac{160 \times \text{Ua Fe}^{55}\ (7.1)}{2{,}300} = 0.497.$$

This value, 0.497, is higher than that of the first post-transfusion sample, 0.418, indicating a loss of about 16 per cent of the transfused cells in the 50-minute interval between starting the transfusion and drawing the first sample.

The lowest recipient Fe^{55} red cell Ua [3] during the first post-transfusion day is used for calculating percentage survival, which is

$$\frac{0.129}{0.497} = 26 \text{ per cent.}$$

It will be noted that the Fe^{59} Ua values of the

[3] No correction for blood loss in sampling was made since the total whole blood drawn, after the transfusion of stored cells, was about 150 ml. or about 3 per cent of the recipient's total blood volume, an average of less than 1 per cent per day.

Protocol Exp. No. GR-78 December 7, 1944

POST-TRANSFUSION SURVIVAL OF WHOLE BLOOD IN ALSEVER'S SOLUTION TRANSFUSED 19 DAYS AFTER DRAWING

Subject: J. G. Age: 28. Sex: male. Height: 183 cm. Weight: 77.2 kgm.

Plasma volume: 3,430 ml. Hct.: 42.1 per cent. Total blood vol.: 5,920 ml. Red cell vol.: 2,490 ml. $\times$ 0.85 = 2,110 ml.

Date	Time	Procedure	Unit activity		Ratio
			Fe^{59}	Fe^{55}	$\frac{\text{Ua } Fe^{55}}{\text{Ua } Fe^{59**}}$
12/7/44	9:45 a.m.	10 mgm. Evans Blue i.v.			
	9:46–	108 ml. whole blood			
	9:48	(44.8 ml. cells) i.v.	1.580		
	9:59	Venous sample	a 0.0340		
	10:10	Venous sample	b 0.0320		
	10:19	Venous sample	c 0.0310		
	1:28 p.m.	Venous sample	d 0.0338		
	1:28–	575 ml. blood at hct. 28 per cent =			
	1:56	160 ml. cells i.v.		7.10	
	2:18	Venous sample	e 0.0319	0.418	13.10
	3:25	Venous sample	f 0.0319	0.335	10.50
	5:30	Venous sample	g 0.0313	0.223	7.08
	9:30	Venous sample	h 0.0337	0.162	5.15
12/8/44	9:42 a.m.	Venous sample	i 0.0318	0.153	4.85
	1:32 p.m.	Venous sample	j 0.0293*	0.129	4.10
12/9/44	9:45 a.m.	Venous sample	k 0.0313	0.156	4.95
	1:00 p.m.	Venous sample	l 0.0323	0.164	5.21
12/10/44	3:35 p.m.	Venous sample	m 0.0332	0.181	5.75
12/11/44	9:45	Venous sample	n 0.0313	0.207	6.57
12/12/44	9:45	Venous sample	o 0.0315	0.255	8.10
		Average	0.0318**		

* Part of sample lost in processing.
** Ua Fe^{59} is average of values for samples e–o, incl., omitting sample j.

recipient red cells remained constant, within the limit of error of the technic, throughout the first 5 days of the experiment, indicating virtually complete retention of these group O tagged cells. The slight fall in Ua samples d and e is to be explained by the additional dilution of the Fe^{59} tagged cells by the transfused Fe^{55} cells.

The ratio of Ua Fe^{55} to Ua Fe^{59} falls progressively during the first 24-hour post-transfusion period and thereafter rises. Since this ratio is an expression of the proportion of Fe^{55} to Fe^{59} tagged cells in the circulation and since virtually all of the Fe^{59} tagged cells were retained, this ratio can also be used for computing percentage of survival.

$$\text{Thus,} \quad \frac{4.1}{15.6} = 26.2 \text{ per cent.}$$

The rise of the ratio after the first post-transfusion day is due to the presence of radio-iron derived from non-viable transfused cells tagged with Fe^{55}.

Figure 3 shows the course of the radioactive red cell level of a subject receiving "tagged" whole blood in McGill II solution, stored under refrigeration for 29 days prior to transfusion. Unit activity data are expressed in terms of percentage of total cells transfuesd Ua/Ua_T. A large proportion of the cells had deteriorated during storage and their removal from the blood stream was reflected by a sharp decline in recipient red cell radioactivity in the first few hours after transfusion, falling to about $\frac{1}{5}$ of the calculated 100 per cent retention value. During the following day this level was unchanged, but thereafter successive samples showed a rise in radioactivity, at first rapid, but leveling off at about 70 per cent of the 100 per cent value.

The question arises as to whether or not those cells which were not rapidly removed from circulation were normally functioning cells with normal life expectancy and remained in circulation throughout the observation period. Denstedt (14) and more recently Thalhimer (15) have shown that when the post-transfusion course of stored cells is followed by the agglutination technic there may be an initial abrupt fall in the recipient's group O cell count, followed by a decreased rate of disappearance, the slope of which closely ap-

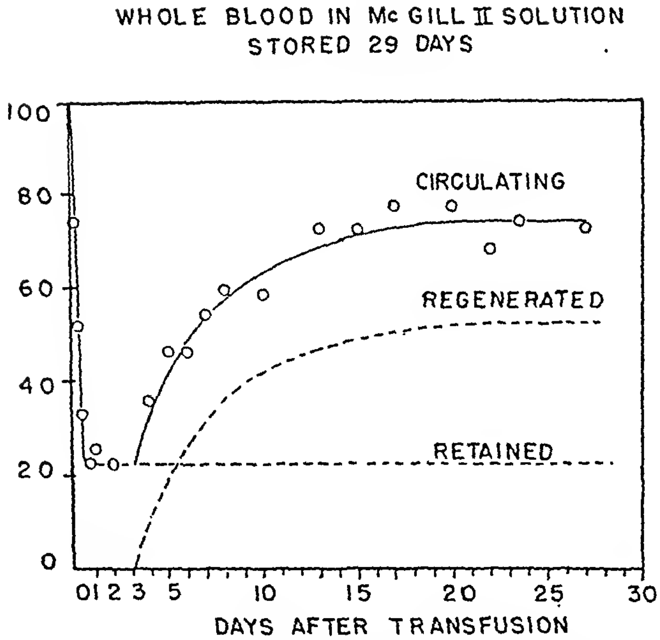

FIG. 3. RE-UTILIZATION OF RADIO-IRON DERIVED FROM NON-VIABLE TRANSFUSED STORED CELLS IN SYNTHESIS OF HEMOGLOBIN

About 80 per cent of the tagged stored cells were rapidly withdrawn from circulation; and the radio-iron contained in these non-viable cells may be considered as available for blood-iron need. For reasons given in the text, it is assumed that those cells not immediately destroyed retain full functional capacity and are retained. Hence, about 20 per cent of the recipient red cell radioactivity is accounted for by these viable cells, and the remainder of the radioactivity, indicated by the broken line, is due to resynthesis into hemoglobin of radio-iron; so about 5/8 of the radio-iron available was re-utilized. From the third post-transfusion day on, the percentage of circulating tagged cells rose, leveling off about the twentieth day at about 75 per cent of the original value.

proximates that obtained for transfused fresh group O cells, somewhere in the neighborhood of 1 per cent per day. This conclusion was confirmed by 2 experiments in which the fate of group O cells tagged with radioactive iron (Fe^{55}) transfused into group A recipients was followed by both the agglutination and radio-iron techniques.

Two such experiments, in which equal aliquots of blood drawn from the same donor into De Gowin's solution were transfused into individual recipients, one on the day of collecting, and the other after storage for 24 days, are illustrated in Figure 4.[4]

Following the transfusion of fresh blood, a prompt initial loss of about 10 per cent of the cells was demonstrated by both methods. Thereafter the number of radioactive cells in circulation increased, reaching the 100 per cent value on the thirtieth post-transfusion day, whereas the count of non-agglutinable cells progressively fell until, the sixtieth day, somewhat less than 40 per cent were detectable in the blood stream. A much greater initial loss occurred in the subject receiving the 24-day-old blood. The immediate (24 to 48 hour) survival was about 40 per cent by the radio-iron and about 70 per cent by the agglutination technic. This discrepancy is probably to be accounted for by a considerable destruction of cells during the interval between the beginning of the transfusion and the taking of the first blood sample for non-agglutinable count. Radioactivity data revealed that 60 per cent of cells had disappeared from the circulation in the first 6 hours after transfusion started. Since the initial post-transfusion count was used as the denominator in calculating percentage of transfused cells remaining in circulation, the computed immediate survival was falsely high.

After the initial loss, a continuous rise in the recipient's red cell radioactivity occurred, reaching a level of about 90 per cent of the 100 per cent value on the fortieth post-transfusion day. During this period the percentage of non-agglutinable cells fell at a rather constant rate.

In the case of both the fresh and the deteriorated blood, however, after the initial drop, the non-agglutinable cells disappeared at a rate of about one per cent per day, or at a mortality rate equal to that of normal cells.

The increase in radioactivity of recipient's cells after the initial decline is due to the presence of tagged cells newly developed since the transfusion. About 80 per cent of the transfused cells were destroyed and their contained iron may be considered as available for re-utilization. The curve of regeneration flattens out at about 50 per cent of the quantity of radioactive cells destroyed during the first day, and hence it is concluded that 5/8 of the total radio-iron from destroyed cells was eventually re-utilized and the remainder either excreted or stored.

The assumption that the retained cells accounted for an almost constant portion of the total recipient cell radioactivity appears to be warranted in this particular experiment, because only young red

[4] These experiments were carried out in collaboration with Dr. Orville Denstedt, McGill University.

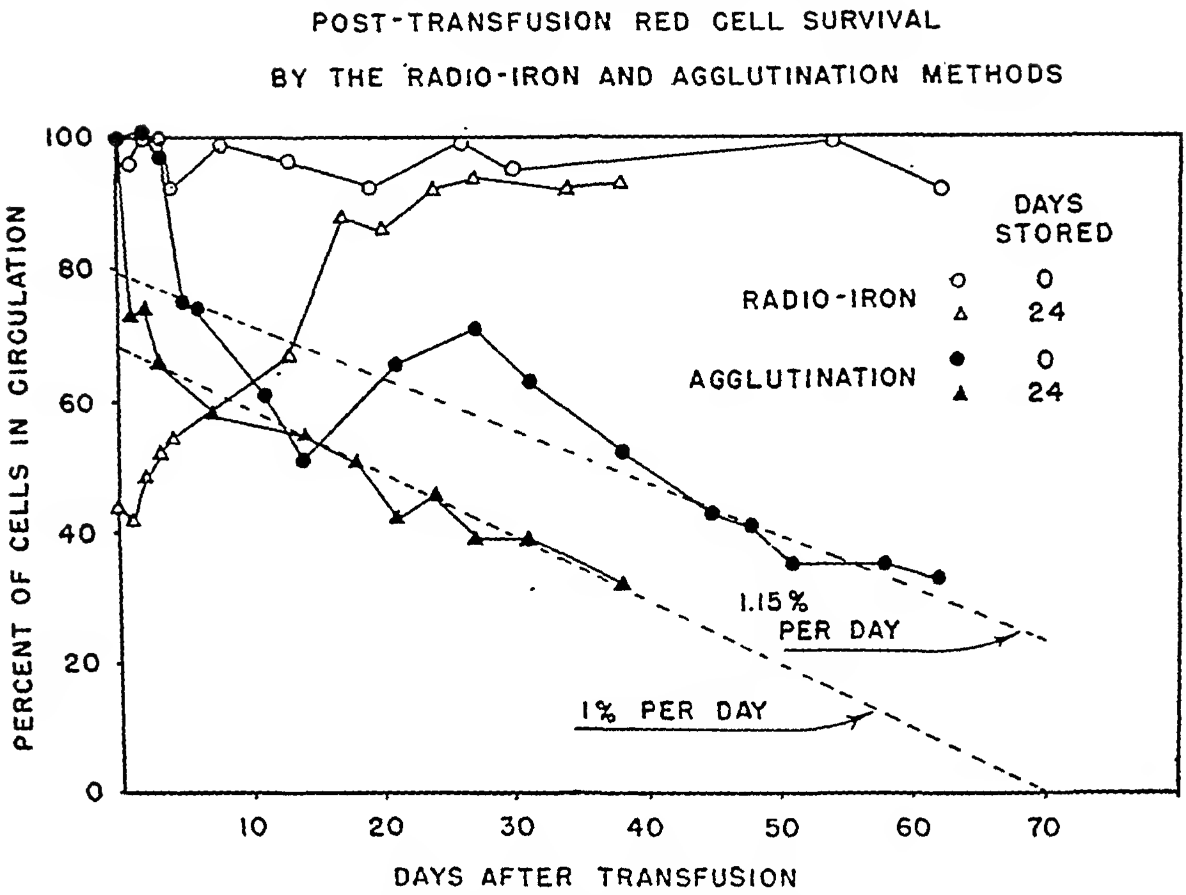

FIG. 4. THE POST-TRANSFUSION SURVIVAL OF GROUP O BLOOD TRANSFUSED FRESH AND AFTER 24 DAYS OF STORAGE INTO AN A RECIPIENT, MEASURED BY BOTH THE RADIO-IRON AND AGGLUTINATION TECHNIQUES

Radioactivity levels clearly show the initial loss of cells and subsequent re-utilization of hemoglobin-derived iron. Non-agglutinable cell counts show an initial loss of non-viable group O cells, and thereafter a continued loss, the rate of which closely approximates the normal red cell mortality rate.

cells had been tagged. The donor blood was drawn 28 days after the donor received his initial injection of radio-iron, and since the blood was stored 29 days, the oldest radio-iron tagged cells were approximately 57 days old when transfused. The normal life span of the adult human erythrocyte is considered to be at least 100 days (19, 20). Hence, the residual cells would remain in circulation during the remaining 27 days of the experiment.

It is important to ascertain whether the behavior of all donor cells, ranging in age from newborn to senescent, was similar during storage. The work of Denstedt (21) and Thalhimer (15), in whose studies the survival of cells representing the entire age population of donor erythrocytes was measured, indicates that the percentage of non-viable cells is roughly proportional to the number of days elapsing between drawing and transfusion. It was therefore necessary to distinguish, radioactively, between young cells and cells that were known to be of mixed age. The known fact of re-utilization of hemoglobin-derived iron was used to advantage to tag differentially young and mixed-age cells.

For example, one donor received the first injection of 5-year iron at 133 days and the last injection 95 days prior to bleeding. He had also received 47-day iron, the first injection 28 days and the last injection 5 days prior to bleeding. Thus, all of the donor's cells tagged with Fe^{59} were young cells, whereas the cells tagged with Fe^{55} were representative of the entire cell population.

Blood from another donor prepared in a similar manner was drawn into acid-citrate-dextrose, and equal aliquots were transfused into 3 recipients after storage for 15, 29, and 41 days, respectively.

Blood from this donor was drawn into ACD, the plasma was removed and the cells resuspended in an acid-citrate-dextrose medium. Aliquots were transfused into 4 recipients after 3, 10, 16, and 30 days' storage at 4° C.

Red cell volume for determination of the 100 per cent retention value was calculated from plasma volume and hematocrit, and percentages of survival were calculated from both the Fe^{55} and Fe^{59} recipient blood levels. In both experiments, as shown in Table I, the survival of the young (Fe^{59}) cells was consistently better than that of the mixed-age (Fe^{55}) cells. The ratio of Fe^{55} to Fe^{59} radioactivities of recipient's cells in relation to the ratio of corresponding radioactivities of the donor's cells was about 0.9, and varied little with the length of storage period.

TABLE I

The comparative survival of young and mixed-age red cells tagged with radio-iron

Days of storage	Day since donor received radio-iron				Percentage survival		Relative retention of mixed-age to young cells*
	Fe^{55}		Fe^{59}		Mixed-age (Fe^{55})	Young (Fe^{59})	
	First	Last	First	Last			
Whole blood in acid-citrate-dextrose							
0	179	153	30	6			
15	193	168	45	21	87	100	0.87
29	208	182	59	35	52	59	0.88
41	230	194	71	47	37	42	0.88
Cells resuspended in acid-citrate-dextrose							
0	133	95	28	5			
3	136	98	31	8	87	100	0.87
10	143	105	38	15	81	93	0.87
16	149	111	44	21	78	83	0.94
30	163	125	58	35	43	46	0.93

* The ratio of Fe^{55} to Fe^{59} radioactivities (Ua) of recipient's cells in relation to the ratio of the same isotopes in a sample of the donor cells transfused.

These experiments indicate that when blood is obtained from donors who have been freshly prepared with radioactive iron, the survival of those cells on storage is proportional to the survival of all the cells, tagged and not tagged. Therefore, a comparison of red cell survival in various preservatives, based on blood from freshly built up donors, truly reflects differences in the value of different solutions.

The use of 2 tracers also permits, under selected conditions, of measurements of changes in the recipient's own cells. In the experiment described in Figure 5, the recipient, blood group A, received 560 ml. of group O blood (220 ml. of cells) of high "anti-A" titre (1/400). Fe^{59} tagged group O cells for cell volume determination were given shortly before, and the value obtained agreed very closely with that calculated from the Fe^{55} recipient levels. During the next 7 days the recipient Fe^{55} and Fe^{59} levels both rose progressively and to about the same degree. At the same time the venous hematocrit progressively fell. It is clear that the proportion of the recipient's cells that were radioactive became greater. Since both lots of group O cells were well retained, as evidenced by the constancy of the ratio of the 2 isotopes, it follows that the rise in radioactivity was due only to destruction of the recipient's own A cells. Calculations based on the original cell volume and the changes in Fe^{55} levels showed a loss of about 700 ml. of A cells. (Calculations based on hematocrit changes showed a loss of only 500 ml.) During the remainder of the observation period the hematocrit rose, and both radioactivity levels declined to almost the original levels, but the ratio of the two isotopes again remained constant. These later changes reflect the regeneration of recipient A cells.

We have applied the method described to the study of a large number of solutions, recommended both for the preservation of erythrocytes as whole blood and as red cell resuspensions after removal of plasma. Results will be reported in subsequent communications.

The method has also been placed at the disposal of several collaborators working in the field of blood preservation. They have built up their own donors with radio-iron prepared in the M.I.T. cyclotron, and donor and recipient blood samples, forwarded by mail, have been chemically prepared and analyzed for radioactivity in our laboratories. Since the cells are destroyed in wet-ashing, the packed cells can be laked prior to shipping. Time in transit or temperature changes do not affect the accuracy of radioactivity measurements.

A standard system of mnemonic symbols for samples of donors' stored and recipients' bloods was developed to facilitate reporting of data to

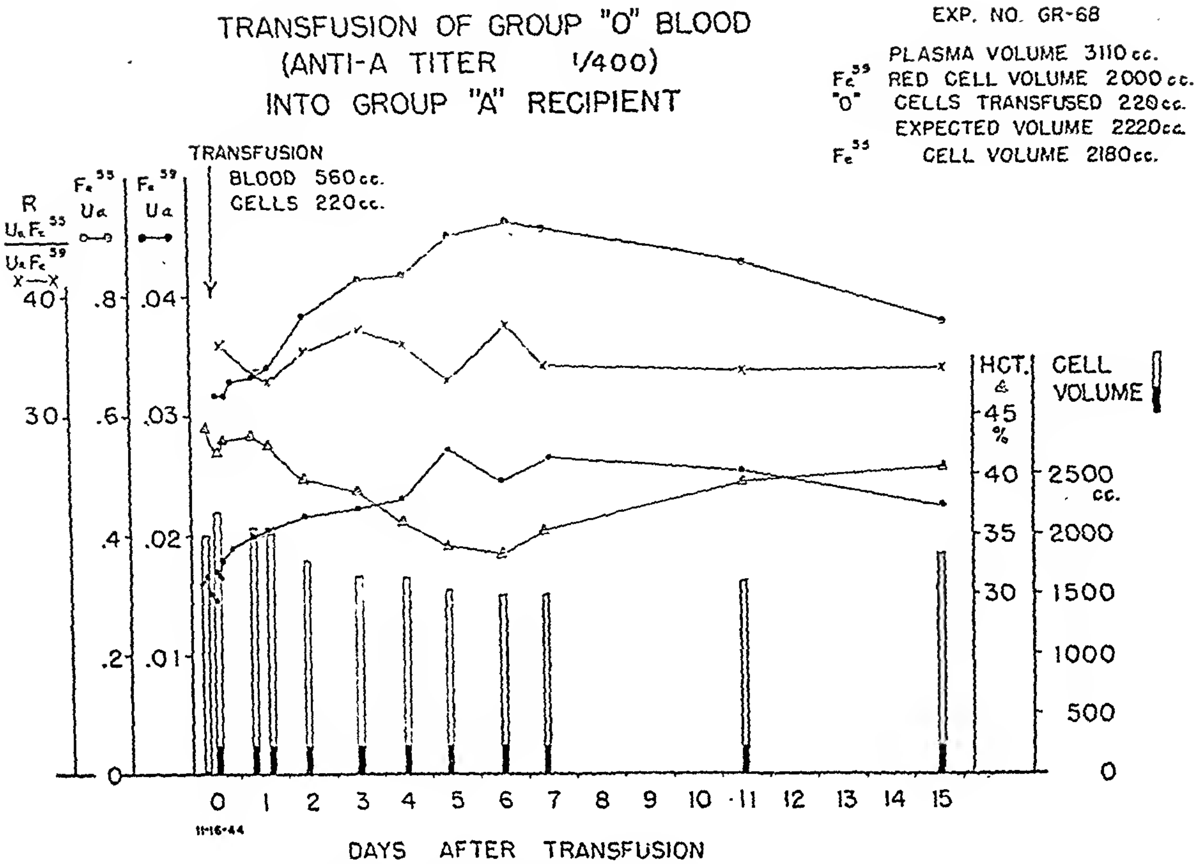

FIG. 5. TRANSFUSION OF HIGH-TITRE ANTI-A GROUP O BLOOD INTO GROUP A RECIPIENT

Recipient's pre-transfusion cell volume as determined by the injection of O cells tagged with Fe^{59} was 2,000 ml., and 220 ml. of stored O cells with Fe^{55} were given. Thereafter the radioactivity level of both isotopes in the recipient's cells rose progressively for 6 days, exceeding the initial post-transfusion values. The ratio of the 2 isotopes, however, remained constant, indicating that both the fresh and stored O cells were all retained. The rise in radioactivity of both isotopes was therefore due to the progressive loss of the recipient's A cells. The recipient's hematocrit fell from an initial value of 43 to a low of 33 in 6 days, and his red cell volume, calculated from Fe^{55} activities, diminished by 700 ml. As recovery occurred, radioactivity levels of both isotopes fell, the hematocrit rose, and red cell volume rose to nearly pre-transfusion level.

collaborators. Because all workers used a common technic, differences in method were reduced to a minimum, and results obtained have been truly comparable.

CONCLUSIONS

(*1*) A method of measuring the post-transfusion survival of preserved stored human erythrocytes by means of 2 radioactive isotopes of iron is described.

(*2*) The method is specific in that only intact circulating cells containing radioactive iron bound in the hemoglobin molecule are detected.

(*3*) The determination of the recipient's pre-transfusion circulating red cell volume by means of fresh group O cells tagged with Fe^{59} permits of accurate calculation of recipient post-transfusion red cell radioactivity level corresponding to 100 per cent retention of transfused stored cells tagged with Fe^{55}. This is important in the study of deteriorated bloods in which red cells may be withdrawn from circulation in the interval between starting the transfusion and obtaining the first post-transfusion blood sample.

(*4*) The extent to which iron derived from non-viable transfused stored cells is re-utilized in the synthesis of new hemoglobin can be measured.

(*5*) The destruction of the homologous cells of patients of group A and B by high anti-A or anti-B titre donor bloods of group O is discussed.

BIBLIOGRAPHY

1. Mollison, P. L., and Young, I. M., Failure of *in vitro* tests as a guide to the value of stored blood. Brit. M. J., 1941, 2, 797.
2. Gibson, J. G., 2nd, and Evans, W. A., Jr., Clinical studies of the blood volume. II. The relation of plasma and total blood volume to venous pressure, blood velocity rate, physical measurements, age and sex in ninety normal humans. J. Clin. Invest., 1937, 16, 317.
3. Ebert, R. V., Stead, E. A., Jr., and Gibson, J. G., 2nd, Response of normal subjects to acute blood loss. With special reference to the mechanism of restoration of blood volume. Arch. Int. Med., 1941, 68, 578.
4. Hahn, P. F., Ross, J. F., Bale, W. F., Balfour, W. M., and Whipple, G. H., Red cell and plasma volumes (circulating and total) as determined by radio-iron and by dye. J. Exper. Med., 1942, 75, 221.
5. Gibson, J. G., 2nd, Seligman, A. M., Peacock, W. C., Aub, J. C., Fine, J., and Evans, R. D., The distribution of red cells and plasma in large and minute vessels of the normal dog, determined by radioactive isotopes of iron and iodine. J. Clin. Invest., 1946, 25, 848.
6. Ebert, R. V., and Stead, E. A., Jr., Demonstration that the cell plasma ratio of blood contained in minute vessels is lower than that of venous blood. J. Clin. Invest., 1941, 20, 317.
7. Gibson, J. G., 2nd, and (by invitation) Seligman, A. M., and Peacock, W. C., The capillary circulation of the normal dog. J. Clin. Invest., 1944, 23, 940.
8. Strumia, M. M., Symposium on new trends in surgery; the fate of transfused refrigerated blood and the problem of blood banks. S. Clin. North America, 1942, 22, 1693.
9. Gilligan, D. R., Altschule, M. D., and Katersky, E. M., Studies of hemoglobinemia and hemoglobinuria produced in man by intravenous injection of hemoglobin solutions. J. Clin. Invest., 1941, 20, 177.
10. Ashby, W., Determination of length of life of transfused blood corpuscles in man. J. Exper. Med., 1919, 29, 267.
11. Wearn, J. T., Warren, S., and Ames, O., Length of life of transfused erythrocytes. Arch. Int. Med., 1922, 29, 527.
12. Mollison, P. L., and Young, I. M., On the survival of the transfused erythrocytes of stored blood. Quart. J. Exper. Physiol., 1941, 30, 313.
13. Maizels, M., and Paterson, J. H., Survival of stored blood after transfusion. Lancet, 1940, 2, 417.
14. Denstedt, O. F., Osborne, D. E., Stansfield, H., and Rochlin, I., The survival of preserved red cells after transfusion. Anesthesiology, 1944, 5, 237.
15. Thalhimer, W., and Taylor, E. S., The transfusion of centrifuged type O cells resuspended and stored in 10 per cent corn syrup. J. A. M. A., 1945, 127, 1096.
16. Ross, J. F., and Chapin, M. A., Effect of storage of citrated blood on the survival of transfused erythrocytes. J. A. M. A., 1943, 123, 827.
17. Peacock, W. C., Evans, R. D., Irvine, J. W., Jr., Good, W. M., Kip, A. F., Weiss, S., and Gibson, J. G., 2nd, The use of two radioactive isotopes of iron in tracer studies of erythrocytes. J. Clin. Invest., 1946, 25, 605.
18. Gibson, J. G., 2nd, Weiss, S., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., Good, W. M., and Kip, A. F., The measurement of the circulating red cell volume by means of two radioactive isotopes of iron. J. Clin. Invest., 1946, 25, 616.
19. Hawkins, W. B., and Whiple, G. H., The life cycle of the red blood cell in the dog. Am. J. Physiol., 1938, 122, 418.
20. Shemin, D., and Rittenberg, D., Studies on the formation of heme and on the average life time of the human red cell. Federation Proceedings, 1946, Vol. 5, Part II, p. 153.
21. Denstedt, O. F., Changes in blood during storage. Canad. M. A. J., 1942, 46, 188.

THE POST-TRANSFUSION SURVIVAL OF PRESERVED HUMAN ERYTHROCYTES STORED AS WHOLE BLOOD OR IN RESUSPENSION, AFTER REMOVAL OF PLASMA, BY MEANS OF TWO ISOTOPES OF RADIOACTIVE IRON [1]

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The modern era of blood transfusions was ushered in by the discovery of human red cell groups by Shattock (1) in 1900 and Landsteiner (2) in 1901. Grouping was later systematized by Jansky (3) and Moss (4). In the course of a few years the paraffined tube method of Kimpton-Brown (5), the multiple syringe technic of Lindeman (6) and the 4-way stopcock apparatus of Unger (7) came into general use in hospitals for direct transfusions. All of these methods required speed in transferring blood from donor to recipient because of the dangers of clotting.

Agote (8) and Lewisohn (9) introduced sodium citrate as an anticoagulant. This avoided the hazards of clotting in the interval between drawing and administering blood and yet permitted the observance of aseptic precautions. The procedure was much simpler than the direct methods which required surgical teams and operating room technic.

The citrate method came into favor very slowly. Early apparatus described by Hoffman (10) and Brines (11) involved suction for collecting and positive air pressure for injecting. Reactions were common. A review of transfusion procedure by Herr as late as 1925 (12) indicated that the direct was still preferred to the citrate method.

The first successful preservation of human whole blood was accomplished in 1916 by Rous and Turner (13) by the addition of dextrose to a sodium citrate anticoagulant. This solution was used to a limited extent in World War I by Robertson (14). Since the ratio of diluent to cells was high, and the amount of citrate large, the supernatant fluid was removed and the cells given in suspension in Locke's solution. This procedure was complicated and laborious and did not come into general use.

A survey of transfusion practice in common use up to 1938 was made by Levine (15). Sodium citrate was the most widely used anticoagulant and blood was discarded after from 5 to 7 days. Little if any use was made of blood preservatives.

There is now little doubt that many reactions attributed to citrate may have been due to pyrogenic contamination of solutions or apparatus. Wechselmann (16) reported febrile reactions following intravenous therapy in 1911, and Seibert in 1923 (17) showed them to be of bacterial origin. Banks (18) demonstrated that pyrogens could be destroyed by autoclaving. Removal of pyrogens by Seitz filtration was reported by Co Tui (19), and by adsorption on charcoal by Lees (20). The control of pyrogens by simple procedures made possible the development of methods for preparing safe intravenous solutions (21) and blood transfusion apparatus (22).

Further knowledge of blood groups (23) tended to reduce reaction rates. The discovery of the significance of the Rh factor by Landsteiner and Wiener (24) explained many untoward transfusion effects.

The advent of economical mechanical refrigerators opened the way for the establishment of blood banks, the first of which, in the U. S. A., was established in 1937 by Fantus (25); others followed promptly (26, 27).

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts Institute of Technology, in collaboration with the Peter Bent Brigham Hospital and the Massachusetts General Hospital.

Interest in better blood preservation was awakened by rumors of World War II and greatly accelerated by the actual advent of hostilities in 1939.

De Gowin and his associates modified the original Rous-Turner solution and studied rates of hemolysis (28), potassium diffusion (29), fragility changes (30), and the effect of temperature (31). They made a clinical study of reactions (32) but did not study post-transfusion survival.

Since then numerous reports on survival as measured by the Ashby agglutination technic (33) have appeared in the literature. Wiener and Schaefer (34) concluded that citrated blood survived poorly after 7 days of storage. Preservation for as long as 18 days was obtained with dextrose-saline-citrate solution studied by Mollison and Young (35). These solutions were difficult to prepare since the dextrose caramelized during autoclaving. This was overcome by acidifying the solution with citric acid. In spite of the very low pH, cell preservation was improved (36).

While the beneficial effect of added dextrose was recognized, the necessary facts upon which to base a military overseas blood program were, in the main, not widely known in 1940. The rate at which cells deteriorated during storage even in the best solutions had not been satisfactorily determined. The rate at which these deteriorated cells broke down in the recipient was not known, and the degree of hemoglobinemia to be expected to follow a transfusion of blood of a given age was a matter of conjecture. The ability of the individual to handle large amounts of plasma hemoglobin was an uncertainty, although the association of hemoglobinuria and renal damage was recognized. There was little knowledge of the degree of normal functional activity retained by those cells not rapidly eliminated after transfusion, nor was it known to what extent the blood-building substance of the destroyed cells would be utilized.

The effect on preservation of mechanical agitation incident to transportation on survival of cells was unknown although De Gowin (37) had shown that spontaneous hemolysis was not increased by shipment by automobile or airplane under refrigeration. Refrigeration was considered necessary, but optimal storage temperatures had not been determined.

Changes in the chemical behavior of the cell during storage in varying concentrations of citrate, saline, and dextrose, at varying hydrogen ion concentrations and temperatures, had not been systematically studied, nor had the effect of these variables on the cell membrane been observed. Various *in vitro* tests had been applied but there was general disagreement as to whether observed *in vitro* behavior bore any relation to the extent to which these changes were reversible when the cell was returned to the blood stream.

In short, the degree to which cells would remain viable in any solution for a given time, under given conditions of transportation and refrigeration, was unknown. And finally, no one could say how bad a transfusion a normal, or more important, a seriously wounded human could withstand. Therefore, the standards of acceptable transfusion of stored blood, or the maximum safe period of storage, could not be defined.

The purpose of the study reported in this and subsequent papers was to investigate the many solutions recommended as preservatives for human erythrocytes, both as whole blood and in resuspension after aspiration of plasma, to ascertain:

(*1*) The percentage of survival of red cells, both as whole blood and in resuspension, after refrigerated storage for varying periods;

(*2*) The rate at which non-viable stored cells are removed from the blood stream and the extent of re-utilization of iron contained in those cells;

(*3*) The effect on survival of depot storage at temperatures ranging from − 4° to + 40° C.; and of transportation by land, sea, and air, with and without controlled refrigeration.

METHODS

The method used in these studies for measuring post-transfusion survival of stored cells by means of 2 radioactive isotopes of iron has been described in previous communications (38, 39, 40). Donor red cells are tagged by the intravenous administration of radioactive iron (Fe^{55}). Since the radioactive iron atoms are firmly bound in the hemoglobin molecule, the presence of donor cells in the recipient's blood stream may be detected as long as the cell remains intact and in circulation. The techniques developed afford high accuracy of measurement. The re-utilization of radio-iron from destroyed cells in new circulating red cells can also be measured. Both the percentage of survival and the rate at which deteriorated cells leave the blood stream in the immediate post-transfusion period can be accurately measured, and hence the method offers a basis for the comparison of preservative solutions.

Recipient pre-transfusion red cell volume was directly measured by transfusion of fresh red cells tagged with Fe^{59} (39), or by the dye method (41). The radioactive blood used was drawn from the donors into ACD–1 from 1 to 48 hours before it was injected, and was refrigerated from the time of the bleeding to shortly before administration. Whole blood containing 25 to 100 ml. of cells (by hematocrit) was aspirated from the collecting bottle with a calibrated syringe and infused directly therefrom. A sample for radioactivity measurement was taken just before administration. The procedure was carried out within a few hours of the receipt of the main transfusion of stored cells.

Experimental subjects[2]

With few exceptions, as noted, all donors and recipients were young male volunteers 18 to 25 years of age. Only donors with negative Wassermann reactions and no history of malaria, jaundice, or other recent infectious disease were used. Donors were of Group O and A, and recipients were of all groups. All recipients and most donors were Rh positive. Donors whose bloods were to be preserved were built up with Fe^{55}; those whose cells were used for recipient red cell volume determinations, with Fe^{59}.

The level of radioactivity induced in individual donors was governed to a great extent by the type of experiments for which their blood was to be used. Thus, if small blood aliquots were to be given, the donor levels were higher, proportionately, than if half or full transfusions were given. Iron dosage was estimated from the nomograms shown in a previous communication (39), so as to yield a minimum of 250 c.p.m. per ml. of recipient's blood. Booster doses were required to maintain adequate levels in donors receiving Fe^{59} (47-day half life).

Many donors were bled repeatedly, but at not less than 8-week intervals. Donors prepared with Fe^{55} (half-life 5 years) maintained the radioactivity red cell level resulting from the initial series of injections for long periods.

Blood collection

Blood was collected either by gravity or in vacuum bottles. During every bleeding, the collecting bottle was gently and continuously agitated. A small sample "pilot tube" of clotted donor blood was obtained at the time of bleeding for cross-matching, checking of blood group, and serologic testing.

Recipients were of all 4 blood groups, but all were Rh positive. Typings were twice checked. In all transfusions of group A blood and in many group O bloods, donor cells were cross-matched with the recipient's serum. When small transfusions were given no preliminary bleeding was done; when the transfusions were large (over 75 ml. of cells) the recipients were usually pre-bled an amount of whole blood at least equal to the quantity they were to receive. Each recipient was used for only 1 transfusion.

Gravity method

Blood from donors whose cells were tagged with Fe^{55} was collected into 500-ml. Fenwal blood bottles containing the anti-coagulant solution. Blood from donors, used for red cell volume measurements, whose cells were tagged with Fe^{59}, was collected into 300-ml. Fenwal blood bottles containing ACD–1 solution.

Vacuum method

Commercially prepared, evacuated bottles of 600 ml. or 1,000-ml. capacity containing sterile, pyrogen-free anti-coagulant, were used.[3] The collecting bottle was inverted during the bleeding so that the blood was drawn up through the anticoagulant solution, and the rate of flow was regulated by means of a screw clamp on the rubber tube, keeping it slow enough to reduce foaming to a minimum.

Subdivision

When aliquots of whole blood from one donor were given to several recipients the subdivision was made shortly after bleeding, portions being transferred from the collecting to smaller storage bottles by gentle suction. Subdivision of all resuspensions was done in like manner, after thorough mixing of cells and diluent. In a few instances multiple aliquots were obtained by bleeding a single donor into several collecting bottles (4 and 6 in Table I).

Resuspension of erythrocytes

In all red cell resuspension experiments the blood was collected by gravity into 500-ml. Fenwal bottles. The period between bleeding and centrifugation varied from 1 to 26 hours. Centrifugation was carried out in the collecting bottle in an unrefrigerated International Model F. centrifuge for 35 minutes at 1,800 to 2,000 r.p.m., and the plasma was removed by suction immediately after centrifugation. In some instances most of the "buffy coat" was removed with the plasma. The resuspension solution was added to the packed cells from ½ to 27 hours after plasma aspiration. During all these time intervals, except during centrifugation, the blood or packed cells were refrigerated. Resuspension solutions were usually chilled before being added to cold packed cells.

All these procedures were carried out in an essentially dust-free room, on a porcelain-topped table, under ultraviolet radiation.

Storage

Whole blood and cell resuspensions were stored at 4 to 6° C. The constancy of the refrigerator temperature range was carefully checked by means of a recording thermometer. Except when briefly removed for culturing, they remained undisturbed until transfused.

Bacteriologic control

Four to 5 days before transfusion, 10 ml. of the whole blood or cell resuspension were aseptically aspirated

[2] Students enrolled in Army A.S.T.P. and Navy V–12 programs and civilian students at Harvard Medical School.

[3] Prepared by Baxter Laboratories, Glenview, Illinois.

TABLE I

Composition of solutions used for preservation of whole blood and for resuspension of packed red cells

Solution	Quantity for 500 ml. whole blood	$Na_3C_6H_5O_7$: $2H_2O$	$C_6H_8O_7$: H_2O	NaCl	NaH_2PO_4	Na_2HPO_4	Conc. HCl. sp. gr. 1.19	H_2CO_3	Na_2HCO_3	NaOH	Dextrose anhydrous	Albumin (Fraction V)	Globulin (Fraction IV-3, 4)		pH
													Lot 151	Lot 301	
(For whole blood)	*ml.*	*grams per 100 ml.*	*grams per 100 ml.*		*grams per 100 ml.*		*ml.*	*grams per 100 ml.*				*grams per 100 ml.*	*grams per 100 ml.*		
Sodium citrate	50	4.00													7.4
McGill II	250	1.280			0.344					0.092	2.160				7.4
DeGowin	450	0.426									4.680				7.4
Alsever	500	0.800	0.0075	0.420							2.050				6.8
Parpart	210	1.50			0.137	0.169					2.36				7.1
ACD-1	125	1.33	0.470								3.00				5.0
ACD-G	75	1.98	0.780								2.30				5.0
Modified E.T.O. citrate	125	1.67		0.830			0.58				2.91				5.0
Modified E.T.O. citrate-dextrose	125	1.46		0.650			0.42				2.08				5.1
(For cell resuspension)	Quantity for 100 ml. of cells														
Isotonic saline	65–75			0.85											7.4
Phosphate-buffered citrate-dextrose	65–75	0.480 0.480		0.440 0.440	0.125 0.310	0.190					1.08 1.08	5.0 5.0			6.8 6.0
Citrate-buffered citrate-dextrose	65–75	0.330	0.100	0.670							1.08				5.1
Modified ACD-1 C_2A C_2A_1	65–75	0.266 0.360	0.094 0.044	0.720 0.690							1.08 1.08				5.0 5.5
Modified ACD (Wash. Conf.)	40	0.120	0.120	0.70							1.80				5.0
Bicarbonate-buffered citrate-dextrose	65–75	0.442		0.228			0.28		0.515		0.553				6.4
Acidified (HCl) citrate-dextrose	65–75	0.480 0.480		0.590 0.590			0.25				1.08 1.08		1.75; 3.50	3.00	7.0 3.8

from the storage bottle. Of this, 2 ml. were inoculated into each of 2 tubes of thioglycolate broth (for both aerobes and anaerobes), and 3 tubes of tryptose phosphate broth (for aerobes). One tube of thioglycolate and one of tryptose broth were incubated at 37° C. in air, one of each at room temperature in air, and the third tryptose tube in 10 per cent CO_2 at 37° C. After 2 to 3 days they were subcultured to similar media and to blood agar plates, and maintained at both body and room temperatures. Examination by smearing and gram staining was done at from 2 to 5 days of incubation.

Transfusion of stored blood

In all experiments, the storage bottle was removed from the refrigerator ½ to 3 hours before the transfusion and *thoroughly mixed* by gentle rotation.

(1) OPEN BURETTE METHOD. All of the blood but 10 or 15 ml. was decanted through 4 layers of cotton gauze into a graduated 300-ml. salvarsan burette, the delivery tube of which had previously been filled with sterile physiologic saline. The 10 or 15 ml. remaining in the storage bottle was used as a representative aliquot for radioactivity measurements and *in vitro* tests.

(2) TRANSFUSION FROM STORAGE BOTTLE. After standard Army expendable recipient sets[4] became available, all transfusions were given directly from the storage bottle. These sets, which were designed for use with the standard vacuum bottle, were easily adapted for use with Fenwal bottles by means of a short length of rubber tubing and a glass vent tube. A representative aliquot of filtered blood for radioactivity and *in vitro* determinations was obtained directly from the delivery tube just prior to the transfusion. The volume administered was determined by water calibration of the collecting bottle.

Radioactivity sampling

TECHNIC. Fifteen ml. recipient blood samples for radioactivity analysis were taken, with a minimum of stasis, into round bottomed graduated 15-ml. hematocrit tubes, using heparin as an anticoagulant. These were spun, stoppered, at 300 r.p.m. for 30 minutes.

SAMPLING SCHEDULES. The recipient samples following transfusion were analyzed for radioactivity of both isotopes of iron. On the day of the tansfusion, samples were obtained 20 minutes, 1 to 1½ hours, and about 4 hours after the transfusion. On the day following the transfusion 2 recipient samples were drawn, several hours apart. In the earlier experiments sampling was carried out to the eighteenth and in some instances to the thirtieth post-transfusion day, a single daily sample being taken for 5 or 6 days after transfusion, followed by triweekly samples. In the later experiments, two samples were obtained on the first and second post-transfusion day, and single daily samples for the next three days, for a total sampling period of six days.

[4] Supplied by Walter Reed Hospital and American Red Cross Blood Donor Service.

Experiments on whole blood anticoagulant-preservatives

The following solutions, the compositions of which are given in Table I, have been studied. (*1*) Four per cent sodium citrate; (*2*) McGill solution, developed and studied by Denstedt (42); (*3*) a modification of the original Rous-Turner solution reported on by De Gowin (43); (*4*) Alsever's citrate-saline-dextrose solution (27); (*5*) a phosphate-buffered citrate-dextrose solution recommended by Parpart (44); (*6*) "ACD–1," and (*7*) ACD–G, modifications of the Loutit-Mollison acid-citrate-dextrose solution studied by Rapoport (45); (*8, 9*) 2 citrate-dextrose solutions routinely supplied to the European Theatre of Operations (Mediterranean Theatre), and (*10, 11*) 2 acidified modifications of the E. T. O. solutions.

In the following description of procedures, "blood" always means blood drawn from a donor previously prepared with radioactive iron (Fe^{55}). Each blood, or blood aliquot, was transfused into an individual who had never before received radioactive iron in any form. All bloods, or aliquots, were stored at 4 to 6° C.

(1) SODIUM CITRATE. OPEN BURETTE METHOD

Experiment Nos. A, B, C, D. Five hundred ml. of blood were taken into 50 ml. of 4 per cent sodium citrate at room temperature from each of 2 donors. One subject received ½ of the blood from the first donor the day it was drawn, and the remainder after storage for 7 days. The blood from the second donor was stored for 7 days, when it was divided into 2 equal portions and transfused. All bloods were prefiltered just prior to administration. The recipients were bled amounts equal to the quantity of citrated blood given just prior to transfusion.

Experiments Nos. 22 through 24. Three hundred ml. of blood were drawn into 30 ml. of 4 per cent sodium citrate from a donor who had received both isotopes of iron. The donor had received his first injection of Fe^{55} 121 days, and of Fe^{59} 24 days prior to bleeding; thus all Fe^{59} tagged cells were young cells. Three aliquots of about 100 ml. each were transfused after storage for 1, 5, and 8 days.

(2) MCGILL SOLUTION. OPEN BURETTE METHOD

Experiments Nos. 1 through 5. The 3 components of the solution were sterilized separately and mixed aseptically just prior to taking the blood. Five hundred fifty ml. of blood were taken at room temperature into 275 ml. of the solution in a Fenwal 1-litre flask, and the total was divided into 5 equal portions. The aliquots were transfused into 5 individuals after storage for 0, 10, 14, 21, and 29 days.

(3) DE GOWIN'S SOLUTION. OPEN BURETTE METHOD

Experiments Nos. 18 through 21. The sodium citrate and dextrose solutions were autoclaved separately and mixed just prior to the taking of blood. Three hundred ml. of blood were drawn into 450 ml. of the solution in a Fenwal 1-litre flask. The entire bleeding was then divided into 4 approximately equal aliquots. The collection and subdivision of blood were carried out at a tempera-

ture not over 6° C. The aliquots were transfused after storage for 0, 13, 23, and 36 days.

(4) ALSEVER'S SOLUTION. OPEN BURETTE METHOD

Experiments Nos. 51, 53, 55. Five hundred ml. of blood were drawn into 500 ml. of Alsever's solution, pH 6.8, in a 1,000-ml. vacuum bottle, and subdivided by gravity into 5 aliquots of about 200 ml. each. Three of these were stored in individual bottles and transfused at 4, 12, and 21 days after drawing. The 2 other aliquots (Experiments Nos. 52 and 54) were used for refrigeration studies, to be reported elsewhere.

(5) WHOLE BLOOD IN BUFFERED CITRATE-DEXTROSE, PH 7.1 (PARPART). OPEN BURETTE METHOD

Experiments Nos. 25 through 27. The citrate and dextrose were autoclaved dry and the phosphate buffer in aqueous solution, in separate containers. Three hundred ml. of blood were drawn by gravity into the dry citrate in a standard collecting bottle. The dextrose was dissolved by adding 130 ml. of the buffer and the dextrose-buffer was then added to the whole blood. After thorough mixing, the entire bleeding was divided into 3 approximately equal aliquots. The taking and subdividing of the blood was carried out at room temperature. Hemolysis of the supernatant plasma was observed on the day after the blood was taken but apparently did not increase on storage. The aliquots were transfused after storage for 0, 14, and 27 days.

(6) ACID-CITRATE-DEXTROSE (ACD–1). OPEN BURETTE METHOD

Experiments Nos. 10 through 13. All 3 components of the solution were dissolved together and autoclaved in a Fenwal blood collecting bottle. Three hundred ml. of blood were drawn by gravity into 75 ml. of the solution, and the total amount was divided into 4 approximately equal aliquots. The collection and subdivision were carried out at not over 10° C. The aliquots were transfused after storage for 2, 11, 20, and 39 days.

Experiments Nos. 32 through 34. The donor from whom this blood was drawn received the first injection of Fe^{55} 133 days and his first injection of Fe^{59} 16 days prior to bleeding. Five hundred ml. of blood were collected into 125 ml. of ACD–1 at room temperature, and divided into 4 approximately equal aliquots, 3 of which were transfused after 15, 29, and 41 days of storage. The fourth aliquot, stored 61 days, was not transfused and became hemolyzed.

(7) ACID-CITRATE-DEXTROSE (ACD-G). STORAGE BOTTLE METHOD

Experiments Nos. 159, 161, 163. Five hundred ml. of blood from each of 3 donors were drawn into 75 ml. of the chilled solution, and, after mixing, each blood was divided into 2 aliquots. One aliquot from each donor was stored as whole blood, the other as packed cells (see Experiments Nos. 160, 162, 164). The whole blood aliquots were transfused at 6, 15, and 22 days after drawing.

Experiments Nos. 165, 167, 169. Five hundred ml. of blood were drawn from each of 3 donors into 75 ml. of the chilled solution, thoroughly mixed, stored at 4° C., and transfused at 12, 19, and 27 days after transfusion. The recipient of the 19-day-old blood was bled 500 ml. prior to transfusion; the other recipients were not bled.

(4 AND 6) COMPARISON OF ACD–1 AND ALSEVER'S SOLUTION PH 6.8. STORAGE BOTTLE METHOD

Experiments Nos. 56, 58, 60 (ACD–1); 57, 59, 61 (Alsever's). The ACD–1 solution was prepared in our laboratories; and sterilized in the collecting bottles. The Alsever's solution was received from Walter Reed Hospital,[5] transferred to the collecting bottles, and then re-sterilized. Six hundred ml. of blood were drawn by gravity from 1 donor; 100 ml. into each of three 200-ml. bottles containing 25 ml. of ACD–1, and 100 ml. into each of 3 bottles containing 100 ml. of Alsever's solution. These aliquots of blood in each solution were transfused after storage for 11, 15, and 21 days.

MODIFICATION OF CITRATE AND CITRATE-DEXTROSE SOLUTIONS USED IN E.T.O. STORAGE BOTTLE METHOD

At the time these experiments were undertaken, 2 citrate solutions were in routine use in E.T.O. (Mediterranean Theatre) for the collecting of 500 ml. of blood: (*1*) 50 ml. of 4 per cent sodium citrate, and (*2*) 70 ml. of 2.5 per cent sodium citrate in 0.85 per cent sodium chloride. It was the practice in E.T.O. to add 30 to 40 ml. of 5 per cent dextrose in isotonic saline to the bottles, after 500 ml. of blood had been drawn into them. The solutions used in our experiments were commercially prepared in vacuum bottles. All recipients were bled 500 ml. within 72 hours prior to transfusion.

(8) E.T.O. CITRATE-DEXTROSE. *Experiments Nos. 101, 103, 113*

(9) E.T.O. CITRATE-SALINE-DEXTROSE. *Experiments Nos. 102, 104, 114*

Five hundred ml. of blood were drawn by vacuum from each of 6 donors; each of 3 bleedings into 50 ml. of 4 per cent sodium citrate; and each of 3 bleedings into 70 ml. of 2.5 per cent sodium citrate in saline. Thirty ml. of 5 per cent dextrose in 0.85 per cent sodium chloride solution were then added with a syringe to each bottle. The bloods in the citrate-dextrose solution were transfused after 4, 9, and 14 days, and those in the citrate-saline-dextrose solution after 4, 9, and 14 days of storage. The 9-day-old blood was placed in a water bath at about 45° C. for 15 minutes before being transfused.

(10) E.T.O. MODIFICATION A. *Experiments Nos. 95, 97, 99*

(11) E.T.O. MODIFICATION B. *Experiments Nos. 96, 98, 100*

Two modifications of the above routine technic were tested. A stock solution of acidified dextrose-saline was prepared, having the following composition:

[5] Through the courtesy of Captain John Reichel, M.C. A.U.S.

	Grams per 100 ml.
Dextrose anhydrous	5.0
NaCl	0.85
1 N HCl	1.0 ml.

This solution had a pH of 4.7, and was autoclaved without caramelization. It was prepared by adding 10 ml. of 1 N hydrochloric acid to 1 litre of the saline dextrose solution in a vacuum bottle.

Modification A. Seventy ml. of the stock solution were added by vacuum to 50 ml. of 4 per cent sodium citrate in the original bottle, with sterile precautions.

Modification B. Fifty ml. of the stock solution were added by vacuum to the 70 ml. of 2.5 per cent sodium citrate in 0.85 per cent sodium chloride in the original bottle, with sterile precautions.

Three full bleedings were taken by vacuum from individual donors into Modification A, and 3 into Modifica-

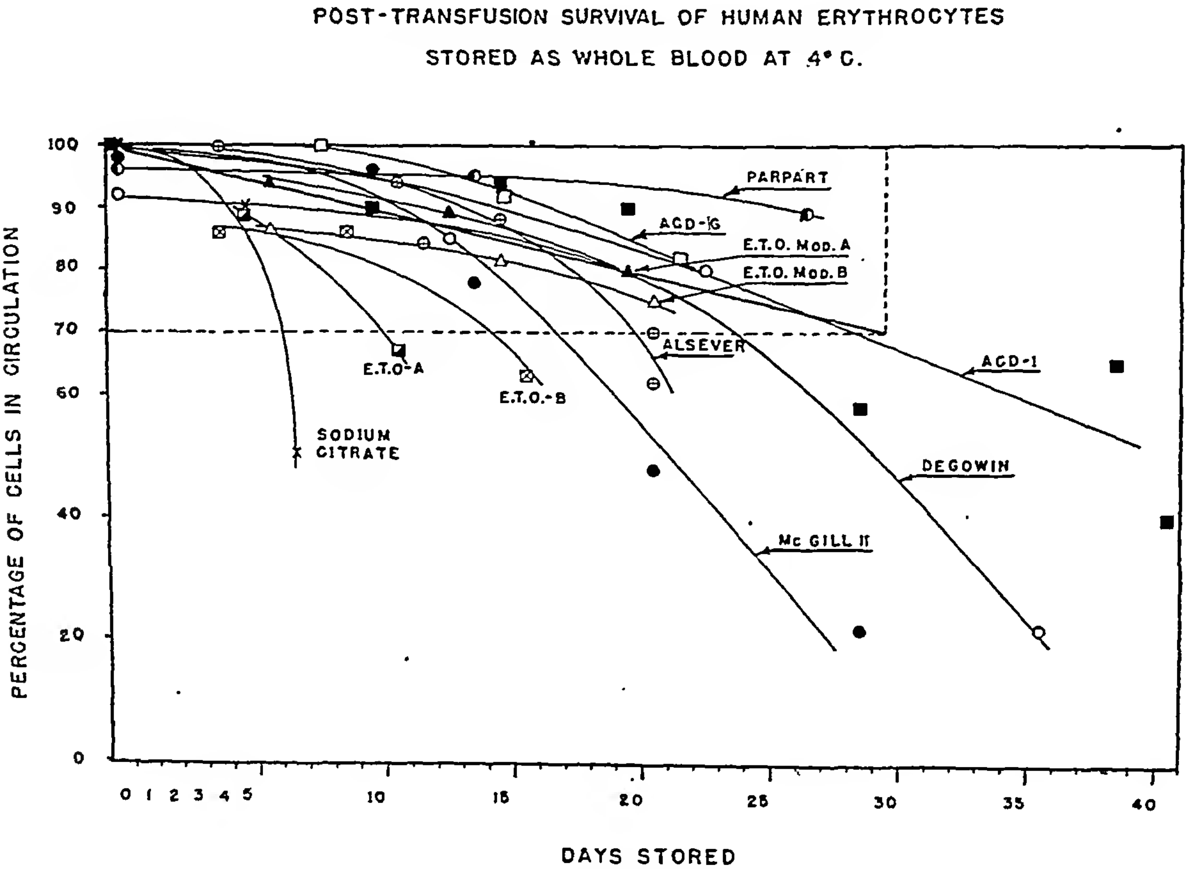

FIG. 1. POST-TRANSFUSION SURVIVAL OF HUMAN ERYTHROCYTES STORED AS WHOLE BLOOD AT 4° C.

In Figures 1, 2 and 3, the solid line represents the normal death rate of live red cells in circulation, approximating one per cent per day. The "zone of safe transfusion" within which 70 per cent or more of the transfused cells are viable is indicated by the broken line rectangle.

Symbols refer to the following solutions:

× = Sodium citrate
○ = De Gowin's solution
◐ = Parpart's solution
● = McGill solution
⊕ = Alsever's solution
■ = ACD-1
□ = ACD-G
◪ = E.T.O. citrate
⊠ = E.T.O. citrate-saline
▲ = E.T.O. Modification A
△ = E.T.O. Modification B

TABLE II

Post-transfusion survival of human erythrocytes drawn as whole blood and stored at 4° C.

Solution	Transfusion no.	Days stored	Blood group: Donor	Blood group: Recip.	Transfused volume: Total	Transfused volume: Cells	Age of tagged cells: Fe^{55}	Age of tagged cells: Fe^{59}	Clots	Reactions (pyrogen)	Survival
					ml.	*ml.*	*days*	*days*			*per cent*
4 per cent sodium citrate (1)	A	0	0	0	337	142	146		++	0	100
	B	0	0	0	335	132	153		++	0	52
	C	7	0	0	250	108	143		++	0	50
	D	8	0	0	268	116	143		++	0	51
	22	1	0	0	112	43	121	24	+	0	100
	23	5	0	0	100	40	121	24	++	0	90
	24	8	0	0	101	40	121	24	++	0	51
McGill (2)	1	0	0	0	157	50	28		+	0	98
	2	10	0	0	142	42	28		++	0	96
	3	14	0	0	117	36	28		++	0	78
	4	21	0	0	137	43	28		++	0	48
	5	29	0	0	143	46	28		++	+	22
DeGowin's (3)	18	0	0	0	157	40	50		+	0	92
	19	13	0	0	166	41	50		+	+	85
	20	23	0	0	141	39	50		++	0	80
	21	36	0	0	153	30	50		++	0	22
Alsever's (4)	51	4	0	A	197	47	83		0	0	100
	53	12	0	0	178	44	83		+	0	84
	55	21	0	A	183	49	83		++	0	70
	57	11	0	A	175	47	37		+	0	94
	59	15	0	A	172	47	37		++	+	88
	61	21	0	0	175	49	37		++	0	62
Parpart's (5)	25	0	0	0	120	34	65		0	0	96
	26	14	0	0	118	33	65		++	0	95
	27	27	0	0	126	39	65		++	0	89
ACD-1 (6)	10	2	0	0	84	37	24		+	0	100
	11	11	0	0	71	31	24		+	0	90
	12	20	0	0	77	35	24		++	0	90
	13	39	0	0	82	37	24		++	0	65
	32	15	0	0	132	51	133	31	+	0	94
	33	29	0	0	128	55	133	45	+	0	58
	34	41	0	0	128	51	133	57	+	0	40
	56	11	0	A	93	42	37		+	0	99
	58	15	0	A	105	48	37		+	0	94
	60	21	0	A	105	48	37		+	0	81
ACD-G (7)	159	8	0	B	269	115	98		0	0	100
	161	15	0	0	249	110	164		++	0	92
	163	22	0	0	268	129	98		+	0	82
	165	12	0	0	545	286	135		+	0	95
	167	19	0	0	546	230	394		++	0	92
	169	27	0	0	459	230	393		++	0	62
E.T.O. 4 per cent citrate (8)	101	4	0	0	610	232	138		++	0	86
	103	9	A	A	595	247	138		++	0	86
	113	14	0	A	540	205	265		++	0	66
E.T.O. 2.5 per cent citrate (9)	102	4	0	0	595	209	144		++	0	89
	114	15	0	0	425	181	208		+++	0	6
E.T.O. Mod. A (10)	95	6	A	A	595	228	86		+	0	94
	97	13	A	A	545	213	118		+	0	89
	99	20	A	A	320	122	116		++	0	80
E.T.O. Mod. B (11)	96	7	A	A	535	202	121		+	0	86
	98	14	A	A	515	186	120		+	0	90
	100	21	A	A	300	120	119		++	0	75

tion B. After the addition of the stock solution, it was still possible to draw about 480 ml. of blood into the bottle without re-evacuation. The bloods in Modification A were transfused after 6, 13, and 20 days of storage, and those in Modification B after 7, 14, and 21 days of storage.

The post-transfusion survival of erythrocytes, preserved as whole blood as described above, is given in Table III and Figure 1.

Experiments on preservative solutions for resuspending red cells after removal of plasma

The composition of the resuspension fluids studied is given in Table I. Except as noted, all red cell resuspensions were from whole blood drawn by gravity into 4 per cent sodium citrate at a 10 to 1 ratio. Bottles were centrifuged at 1,900 r.p.m. for 30 minutes. The quantity of resuspension fluid added to the packed red cells was approximately equal to the amount of plasma removed, except in the case of the modified acid citrate (Washington Conference—Experiments Nos. 120 through 123).

Two types of fluids were used: (*1*) solutions of electrolytes with and without dextrose, and (*2*) electrolyte-dextrose solutions to which human serum protein fractions had been added. All donors were blood group O.

Electrolyte solutions

(12) ISOTONIC (0.85 PER CENT) SODIUM CHLORIDE. OPEN BURETTE METHOD

Experiments Nos. 6 through 8. Five hundred ml. of citrated blood were centrifuged, and the cells were resuspended in 0.85 per cent sodium chloride, within 2 hours of the time of collection. All procedures were carried out at room temperature. This cell resuspension was divided into 3 equal portions and transfused after 0, 3, and 10 days' storage.

(13) TEN PER CENT CORN SYRUP. OPEN BURETTE METHOD

Experiments Nos. 14 through 17. The corn syrup used was supplied by Dr. Thalhimer[6] (46). Three hundred fifty ml. of whole blood were drawn into 35 ml. of 3.2 per cent sodium citrate and centrifuged in a refrigerated centrifuge. The plasma was removed, and an equal volume of corn syrup was added to the cells, within 2 hours of the time of blood collection. The entire procedure was carried out at not over 10° C. After thorough mixing, the cell resuspension was divided into 4 equal aliquots, and these were transfused after storage at 4° C. for 0, 5, 12, and 21 days.

[6] The composition is said to be as follows:

	Grams per 100 ml.
Dextrose	17.7
Maltose	16.8
Higher sugars	16.2
Dextrins	29.6
Moisture	19.7
The pH of this solution is	4.6

STORAGE BOTTLE METHOD

Experiments Nos. 140 through 147. A more extensive series of experiments was conducted in collaboration with Dr. Thalhimer and Lt. H. Blake, M.C., U.S.N.R. Full bleedings were taken by gravity from each of 8 donors, the plasma was removed, and the cells were resuspended in chilled 10 per cent corn syrup within 24 hours of collection. The bottles were shipped in a portable refrigerator by truck to New York City. They were stored in the refrigerator of the New York Red Cross Blood Donor Center. These cell resuspensions were transfused into selected patients on the wards of the New York Hospital at 9, 11, 12, 13, 14, 15, 18, and 20 days after drawing.

(14) PHOSPHATE-BUFFERED CITRATE-DEXTROSE SOLUTION, PH 6.8 (HUGHES). OPEN BURETTE METHOD

Experiments Nos. 28 through 31. The red cells from 320 ml. of whole citrated blood were resuspended, at room temperature, at about 20 hours after the blood was drawn. After thorough mixing, the entire cell resuspension was divided into 4 approximately equal aliquots, which were transfused after storage for 2, 6, 11, and 20 days.

(15) PHOSPHATE-BUFFERED DEXTROSE SOLUTION. PH 6.0 (HUGHES). OPEN BURETTE METHOD

Experiments Nos. 42 through 45. The donor from whom this blood was drawn received the first injection of Fe^{55} 133 days and his last injection of Fe^{55} 95 days prior to the bleeding for this experiment. His first and last injections of Fe^{59} were 16 and 5 days, respectively, prior to bleeding.

Five hundred ml. of blood were collected into 125 ml. of ACD–1 at room temperature. This was stored for about 40 hours at 4° C., and the cells were then resuspended. This cell resuspension was divided into 4 approximately equal aliquots, which were transfused at 3, 10, 16, and 30 days of storage.

(16) CITRATE-BUFFERED CITRATE-DEXTROSE SOLUTION, PH 5.0 (HUGHES). STORAGE BOTTLE METHOD. (This series was for a direct comparison with 10 per cent corn syrup, Experiments Nos. 140 through 147)

Experiments Nos. 132 through 139. The red cells from eight 500-ml. bleedings were resuspended in the chilled solution about 24 hours after collection. The bottles were shipped to New York in the same portable refrigerator used for the cells resuspended in 10 per cent corn syrup (Experiments Nos. 140 through 147), and stored under identical conditions. These resuspensions were transfused into ward patients at the New York Hospital after 10, 11, 13, 14, 15 (2), 18, and 20 days after drawing.

(17) CITRATE-BUFFERED CITRATE-DEXTROSE SOLUTION, PH 3.8 (HUGHES). COLLECTING BOTTLE METHOD

Experiments Nos. 89, 91, 93, 105, 107. Three full bleedings were taken from each of 3 donors, the cells were resuspended in the chilled solution within 24 hours

and transfused after 10, 18 and 22 days of storage. One full bleeding from a fourth donor was resuspended, mixed, and subdivided into 2 aliquots which were transfused after 32 and 39 days of storage.

Experiments Nos. 109 through 112. Five hundred ml. of blood, drawn in equal amounts into 2 collecting bottles, were taken from 2 donors, and the cells were resuspended in the collecting bottles within 2½ hours from the time of bleeding. The solution was at room temperature. Two aliquots from one donor were transfused after 8 and 21 days, and the aliquots from the other donor after 15 and 28 days of storage.

(18 AND 19) MODIFIED ACD (RAPOPORT C_2A, C_2A^1). OPEN BURETTE METHOD

Experiments Nos. 46 through 49. Five hundred ml. of citrated blood from 1 donor were divided into 2 equal aliquots. Each of these was in turn divided into 2 equal aliquots, centrifuged, and the cells resuspended, 2 in C_2A and two in C_2A^1. The aliquots resuspended in C_2A were transfused after 13 and 20 days, and those resuspended in C_2A^1 were transfused after 13 and 22 days of storage.

(20) MODIFIED ACID CITRATE.[7] STORAGE BOTTLE METHOD

Experiments Nos. 120 through 123. Five hundred ml. of blood were taken by gravity from 2 donors into sodium citrate. The blood from each donor was drawn into 2 Fenwal collecting bottles, so that each bottle contained 250 ml. of blood. Within 24 hours from the time of collection, the cells in each bottle were resuspended in only 50 ml. of the solution, which was pre-chilled. These resuspensions were transfused after 6, 10, 16, and 21 days of storage. The tagged cells in the 6- and 10-day stored resuspension were no more than 46 days old, while those in the 16- and 21-day stored resuspensions were of mixed age, on the day of transfusion.

(21) BICARBONATE-BUFFERED CITRATE-DEXTROSE, PH 6.5 (HUGHES). STORAGE BOTTLE METHOD

Experiments Nos. 155 through 158. Five hundred ml. of whole blood were taken from each of 2 donors into 125 ml. of ACD–1. About 20 hours after collection, bloods were centrifuged and plasma removed in the usual manner, the packed cells being refrigerated for 4 hours. Three hundred ml. of chilled diluent were then added to each, by siphonage (to prevent escape of CO_2). Each cell resuspension was then divided into 2 aliquots, by gentle suction. The aliquots were transfused after 6, 13, and 21 days of storage at 4° C.

The results of these experiments are summarized in Table III and Figure 2.

(22) PACKED CELLS FROM WHOLE BLOOD IN ACD–1. STORAGE BOTTLE METHOD

Experiments Nos. 115 through 119. Five hundred ml. of blood were taken from each of 5 donors into 125 ml. of cold ACD–1 by gravity. After 24 hours of refrigeration, the bottles were centrifuged, the plasma removed, and the bottles placed in storage. The packed cells were transfused after storage for 9, 14, 15, 16, and 21 days. The cells stored 15 days (No 115) were transfused as packed cells, without the addition of saline. In the other 4 experiments, 125 ml. of sterile normal saline were added ½ hour before the blood was transfused. The cells were thoroughly mixed with the diluent before administration.

(23) PACKED CELLS IN ACD–G. COLLECTING BOTTLE METHOD

Experiments Nos. 160, 162, 164. No added diluent. Five hundred ml. of blood were taken by gravity from 1 donor, and subdivided as in Experiments Nos. 159, 161, and 163, [see (7) above]. The collecting bottles were centrifuged, the plasma was withdrawn, and the packed cells were transfused after 7, 14, and 21 days of storage, without the addition of any diluent.

Experiments Nos. 166, 168, 170. Diluent added at time of transfusion. Five hundred ml. of blood were taken from each of 3 donors. The collecting bottles were centrifuged, the plasma was withdrawn, and the packed cells were transfused after 12, 19, and 27 days of storage. One hundred twenty-five ml. of 0.85 per cent NaCl were added just prior to transfusion.

Experiments Nos. 176 and 175. 0.86 per cent NaCl + 0.5 per cent dextrose. Two hundred fifty ml. of blood were taken from each of 2 donors. One hundred ml. of diluent were added. The resuspensions were transfused after storage for 14 and 20 days. Hematocrits were 50.5 and 56.4. Survival was 95 and 84 per cent.

Experiments Nos. 171 and 172. 1.0 per cent NaCl. Two hundred fifty ml. of blood were taken from each of 2 donors. One hundred ml. of diluent were added. The resuspensions were transfused after storage for 13 and 21 days. Hematocrits were 47 and 50. Survival was 100 and 86 per cent.

Experiments Nos. 173 and 174. 1.0 per cent NaCl + 0.5 per cent dextrose. Two hundred fifty ml. of blood were taken from each of 2 donors. One hundred ml. of diluent were added. The resuspensions were transfused after 14 and 20 days of storage. Hematocrits were 48 in both instances. Survival was 100 and 73 per cent.

Experiments Nos. 178 and 177. 10 per cent corn syrup. Two hundred fifty ml. of whole blood were taken from each of 2 donors. One hundred ml. of diluent were added. The resuspensions were transfused after 14 and 21 days of storage. Hematocrits were 53.5 and 64.5. Survival was 64 and 81 per cent.

Experiments Nos. 180 and 181. Stored with diluent added. *1.1 per cent NaCl + 0.5 per cent dextrose.* Two hundred fifty ml. of blood were taken into each of 2 bottles from the same donor. One hundred ml. of diluent were added immediately after removal of plasma. The resuspensions were transfused after 12 and 19 days of storage.

Experiments Nos. 182 and 183. Stored with diluent added. *0.85 per cent NaCl + 0.5 per cent dextrose.* Two

[7] Solution recommended for trial at Conference on Resuspended Blood Cells, of C.M.R., at Washington, D. C., 5/18/45.

TABLE III

Post-transfusion survival of human erythrocytes drawn as whole blood in 4 per cent sodium citrate and stored after resuspension in electrolyte solutions at 4° C

Resuspension solution	Transfusion no.	Days stored	Recip. blood group	Transfused volume: Total	Transfused volume: Cells	Age of tagged cells*: Fe^{55}	Age of tagged cells*: Fe^{59}	Clots	Reactions	Survival
				ml.	*ml.*	*days*	*days*			*per cent*
0.85 per cent sodium chloride (12)	6	0	0	175	68	40		0	0	92
	7	3	0	160	68	40		+++	0	90
	8	10	0	140	64	40		++++	0	8
10 per cent corn syrup (13)	14	0	0	59	31	24		0	0	100
	15	5	0	57	19	24		+	0	60
	16	12	0	62	20	24		+	0	42
	17	21	0	97	32	24		++	0	17
	140	9	0	502	227	9		+	+	95
	141	13	A	487	169	9		+	0	94
	142	15	A	475	178	11		+	0	41
	143	20	A	470	193	11		0	0	45
	144	11	0	479	189	8		+	0	92
	145	12	A	480	173	12		+	0	78
	146	14	A	520	220	10		+	0	60
	147	18	0	425	164	10		0	0	58
Phosphate-buffered citrate-dextrose pH 6.8 (14)	28	2	0	74	30	88		+	+	98
	29	6	0	60	24	88		+	0	97
	30	11	0	67	26	88		+	0	88
	31	20	0	64	23	88		+	0	86
Phosphate-buffered citrate-dextrose pH 6.0 (15)	42	3	0	134	58	133	28	+	0	90
	43	10	A	137	58	133	28	+	0	94
	44	16	A	131	56	133	28	+	0	80
	45	30	A	117	50	133	28	++	+	45
Citrate-buffered citrate-dextrose pH 5.0 (Hughes) (16)	132	10	B	503	232	341		+	0	93
	133	13	0	467	217	8		0	0	70
	134	15	0	520	235	343		0	0	82
	135	20	A	315	147	403		++	0	72
	136	11	0	510	256	10		0	0	54
	137	14	0	500	234	10		0	0	40
	138	15	A	492	245	10		+	0	74
	139	18	A	357	175	0		++	++	31
Citrate-buffered citrate-dextrose pH 5.8 (Hughes) (17)	89	10	0	472	182	179		+	+	87
	91	18	0	420	191	104		+	0	85
	93	22	0	300	137	107		+	0	80
	105	31	B	200	96	251		+	0	45
	107	38	0	165	81	251		++	0	0
	109	8	B	260	120	237		+	0	92
	110	15	0	270	130	160		+	+	85
	111	21	0	272	129	237		+	0	76
	112	28	0	237	109	160		+	0	27
Mod. ACD (Rapoport) C_2A, pH 5 (18)	46	13	A	142	46	216		+	+	83
	47	20	A	92	42	216		+	+	62
Mod. ACD (Rapoport) C_2A^1, pH 5.5 (19)	48	13	A	107	50	216		+	+	90
	49	22	A	107	50	216		+	+	69
Mod. ACD (Washington Conf.) (20)	120	6	0	157	104	36		+	0	94
	121	10	A	160	106	36		+	0	80
	122	16	A	138	94	231		+	0	58
	123	21	0	113	49	231		+	0	32
Bicarbonate-buffered citrate-dextrose (21)	155	6	0	259	100	337		+	0	90
	156	13	A	273	115	337		+	0	89
	157	19	0	246	100	337		+	0	71
	158	28	0	256	109	337		0	0	46

* Days between last injection of radioactive iron and day of bleeding.

hundred fifty ml. of blood were taken into each of 2 bottles from the same donor. One hundred ml. of diluent were added immediately after removal of plasma. The resuspensions were transfused after 13 and 20 days of storage.

Experiments Nos. 184 and 185. Stored with diluent added. *0.6 per cent NaCl. + 0.5 per cent dextrose.* Two hundred fifty ml. of blood were taken into each of 2 bottles from the same donor. One hundred ml. of diluent were added immediately after removal of plasma. The resuspensions were transfused after 13 and 20 days of storage.

Experiments Nos. 186 and 187. Stored with diluent added. *0.86 per cent NaCl + 0.5 per cent dextrose + 0.2*

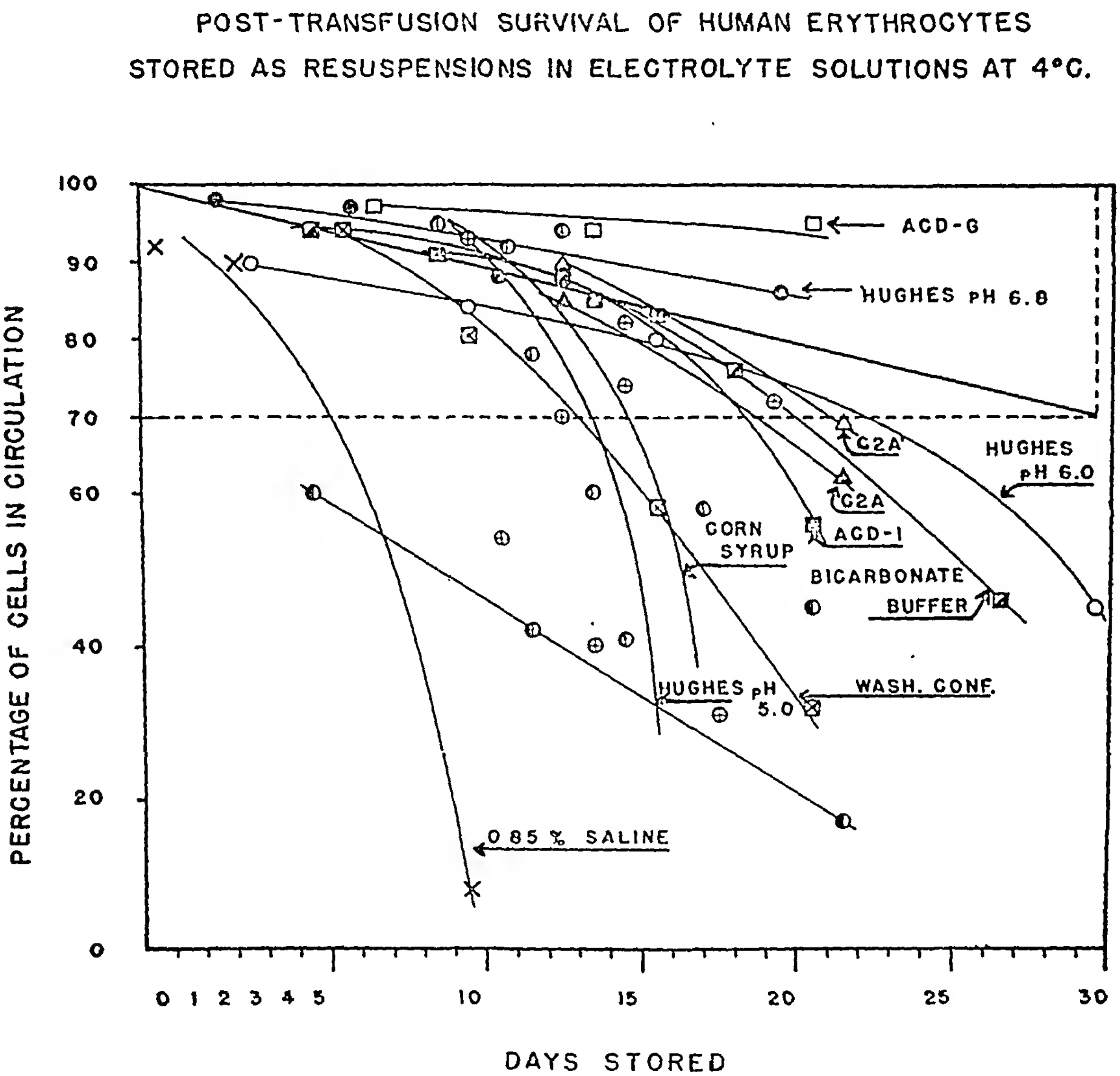

FIG. 2. POST-TRANSFUSION SURVIVAL OF HUMAN ERYTHROCYTES STORED AS RESUSPENSIONS IN ELECTROLYTE SOLUTIONS AT 4° C.

Symbols refer to the following solutions:

× = Isotonic saline
⊕ = 10 per cent corn syrup
● = Phosphate-buffered citrate-dextrose (Hughes) pH 6.8
○ = Phosphate-buffered citrate-dextrose (Hughes) pH 6.0
◐ = Citrate-buffered citrate-dextrose (Hughes) pH 5.0
▲ = Modified ACD (C_2A)
△ = Modified ACD (C_2A^1)
⊠ = Modified ACD (Washington Conference)
◪ = Bicarbonate-buffered citrate-dextrose.

The symbols ■ and □ refer to packed cells in ACD-1 and ACD-G stored without resuspension.

TABLE IV

Post-transfusion survival of human erythrocytes drawn as whole blood in acid-citrate-dextrose and stored at 4° C. with and without resuspension after removal of plasma

Preservative solution	Trans-fusion no.	Days stored	Recip. blood group	Diluent added	Quantity added	Transfused volume Total	Transfused volume Cells	Clots	Reactions	Survival
					ml.	*ml.*	*ml.*			*per cent*
Diluent added at time of transfusion										
ACD-1 (22)	115	15	A	0.85 per cent NaCl	0	158	138	+	0	76
	116	16	A	0.85 per cent NaCl	125	425	226	0	0	83
	117	9	A	0.85 per cent NaCl	125	345	227	+	0	91
	118	14	0	0.85 per cent NaCl	125	363	232	0	0	85
	119	21	A	0.85 per cent NaCl	125	278	161	+	0	56
ACD-G (23)	160	7	B	0.85 per cent NaCl	0	141	122	0	0	97
	162	14	0	0.85 per cent NaCl	0	138	119	++	0	94
	164	21	0	0.85 per cent NaCl	0	102	87	++	0	81
	166	12	0	0.85 per cent NaCl	125	353	236	0	0	98
	168	19	0	0.85 per cent NaCl	125	368	242	++	0	67
	170	27	B	0.85 per cent NaCl	125	335	197	++	0	46
	171	13	0	1 per cent NaCl	100	255	128	+	0	100
	172	21	0	1 per cent NaCl	100	229	108	0	0	86
	173	14	B	1 per cent NaCl + 0.5 per cent dext.	100	234	112	0	0	100
	174	22	A	1 per cent NaCl + 0.5 per cent dext.	100	215	103	+	0	73
	176	14	B	0.86 per cent NaCl + 0.5 per cent dext.	100	222	112	+	0	95
	175	20	0	0.86 per cent NaCl + 0.5 per cent dext.	100	220	124	+++	0	87
	178	15	0	10 per cent corn syrup	100	204	128	+	0	64
	177	21	A	10 per cent corn syrup	100	220	135	++	0	81
Stored with diluent added										
ACD-G (23)	180	12	A	1.1 per cent NaCl + 0.5 per cent dext.	100	215	104	++	0	92
	181	19	0	1.1 per cent NaCl + 0.5 per cent dext.	100	245	118	++	0	78
	182	13	0	0.85 per cent NaCl + 0.5 per cent dext.	100	216	115	++	0	83
	183	20	A	0.85 per cent NaCl + 0.5 per cent dext.	100	213	109	+	0	69
	184	13	A	0.6 per cent NaCl + 0.5 per cent dext.	100	223	125	+	0	71
	185	20	0	0.6 per cent NaCl + 0.5 per cent dext.	100	236	130	+	0	64
	186	14	A	0.86 per cent NaCl + 0.5 per cent dext. + 0.2 per cent citric acid	100	269	149	+++	0	62
	187	21	0	0.86 per cent NaCl + 0.5 per cent dext. + 0.2 per cent citric acid	100	211	112	+	0	48

per cent citric acid. Two hundred fifty ml. of blood were taken into each of 2 bottles from the same donor. One hundred ml. of diluent were added immediately after removal of plasma. The resuspensions were transfused after 14 and 21 days of storage.

The results of experiments on packed cells are summarized in Table IV and shown in Figures 2 and 4.

Experiments on electrolyte solutions with added human serum protein fractions[8]

The albumin used, Fraction V, was the commercial product supplied to the U. S. Navy. The globulins, Frac-

[8] The human serum proteins were obtained through the courtesy of Dr. E. J. Cohn.

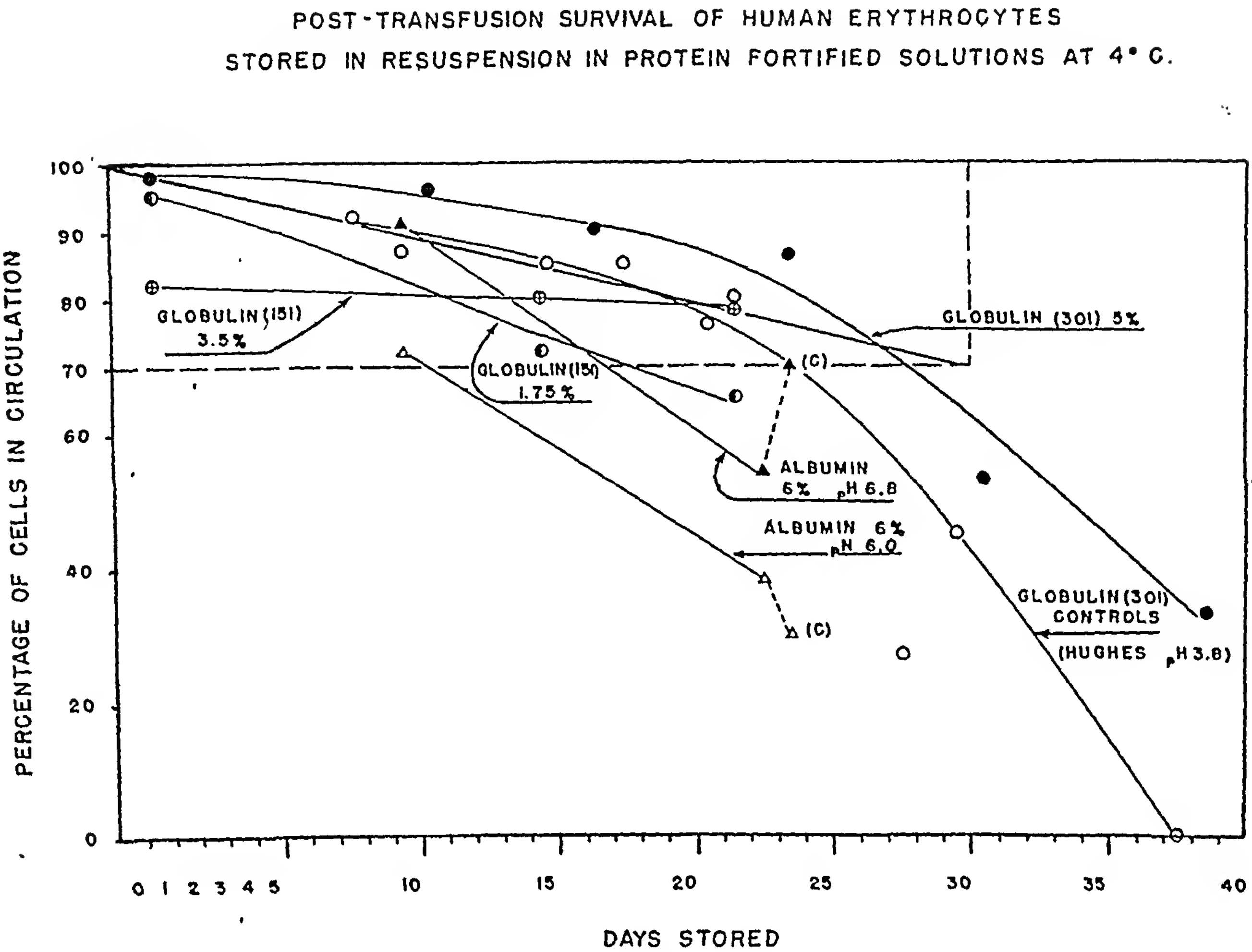

Fig. 3. Post-Transfusion Survival of Human Erythrocytes Stored in Resuspension in Protein-Fortified Solutions at 4° C.

The symbols refer to the following solutions:

▲ = Human serum albumin added to phosphate-buffered citrate-dextrose at pH 6.8.

△ = Human serum albumin added to phosphate-buffered citrate-dextrose at pH 6.0.

⊕ ◐ = Human serum globulin (Lot 151) added to citrate-buffered citrate-dextrose, pH 7.0.

● = Human serum globulin (Lot 301) added to citrate-buffered citrate-dextrose, pH 3.8.

○ = The globulin-free controls for globulin 301.

tion IV-3, 4,[9] were prepared in the pilot plant of the Department of Physical Chemistry, Harvard Medical School. Red cells resuspended in a solution, of the same composition as that to which the proteins were added, were used for control transfusions.

(24 AND 25) HUMAN SERUM ALBUMIN (FRACTION V) IN PHOSPHATE-BUFFERED CITRATE-DEXTROSE SOLUTIONS AT PH 6.8 AND 6.0. OPEN BURETTE METHOD

Experiments Nos. 36, 37 (6.8), Experiments Nos. 39, 40 (6.0), and Experiments Nos. 38 and 41 (controls). With venesection the donor was bled into four 250-ml. storage bottles, designated as bottles (a), (b), (c), and (d). Bottles (a) and (b) each contained 20 ml. of 4 per cent sodium citrate solution, and each received 200 ml.

[9] Safety tests on Fraction IV-3, 4 were carried out by Dr. O. Krayer, in animals, and by Dr. C. A. Janeway, in humans.

of donor blood. Bottles (c) and (d) each contained 10 ml. of 4 per cent sodium citrate solution, and each of these received 100 ml. of the blood. These were stored at 4° C. over night, and then all 4 bottles were centrifuged, and the plasma was removed. To each bottle of packed cells was added a volume of resuspension solution approximately equal to the volume of plasma withdrawn. Bottle (a) received albumin solution buffered to a pH of 6.8. Bottle

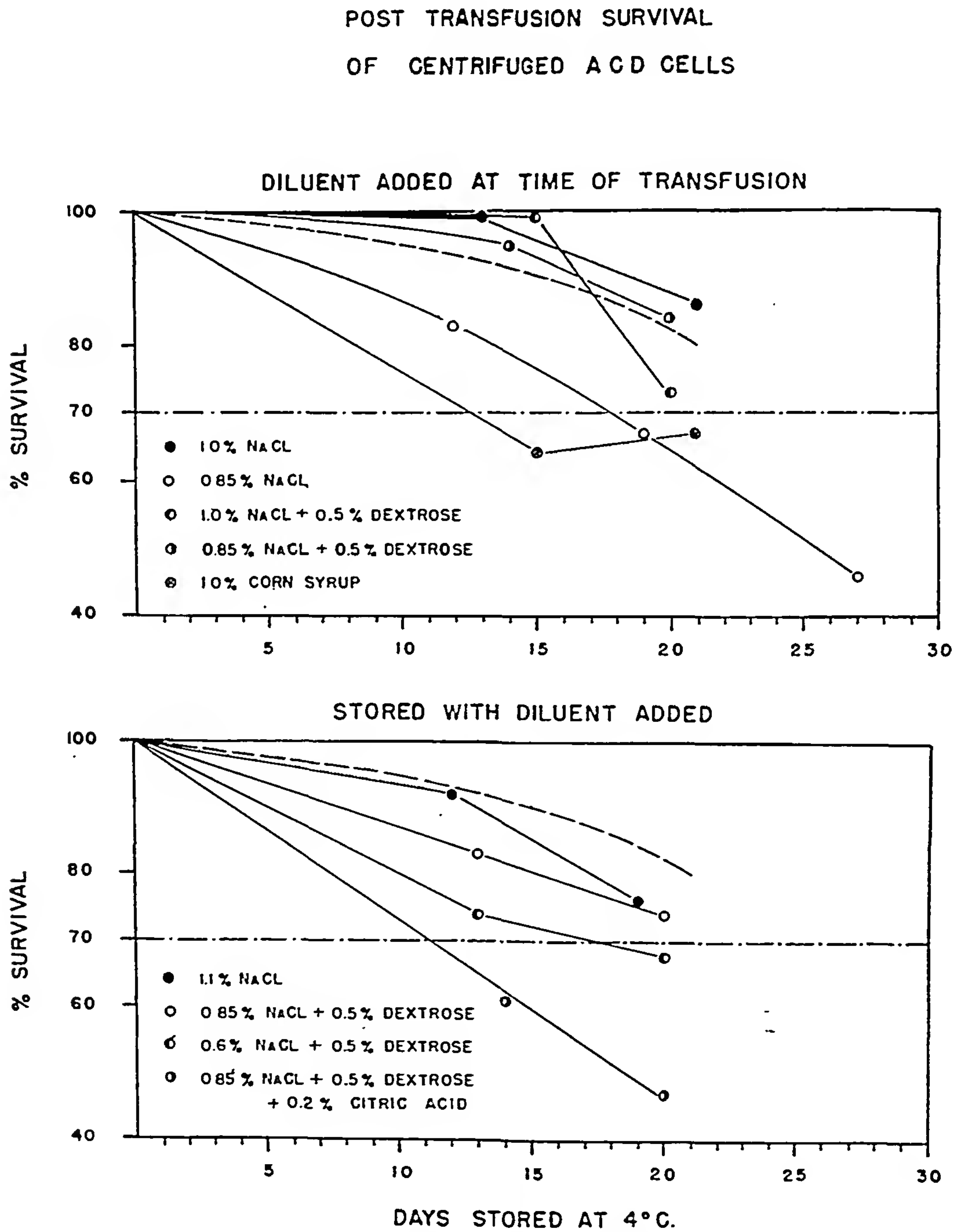

FIG. 4. POST-TRANSFUSION SURVIVAL OF CENTRIFUGED ACD CELLS

The addition of a slightly hypertonic saline solution just prior to transfusion does not decrease the viability of packed red cells drawn as whole blood in ACD. Storage in these same diluents increases the rate of deterioration.

(b) received albumin solution buffered to a pH of 6.0. Bottle (c) received albumin-free solution buffered to a pH of 6.8, and bottle (d) received albumin-free solution buffered to a pH of 6.0. Bottles (a) and (b) were each subdivided into 2 equal aliquots. The entire procedure was carried out at room temperature. After 10 days of storage, an aliquot of cells resuspended in albumin pH 6.8 and one of cells resuspended in albumin pH 6.0 were transfused. After 23 storage days the 2 remaining albumin-containing aliquots were transfused. The control aliquots, that is, *cell resuspended in a solution buffered to 6.8 and 6.0 (no albumin)*, were both transfused after 24 days of storage.

(26) α AND β GLOBULINS (FRACTION IV–3, 4, LOT 151) IN ACIDIFIED-CITRATE-DEXTROSE (HUGHES PH 3.8)

Experiments Nos. 82 through 87. The globulin was dissolved in the electrolyte solution, in 2 portions, the final concentration of protein being 1.75 and 3.50 grams per 100 ml. Five hundred ml. of blood from each of 2 donors were drawn into 4 per cent sodium citrate at room temperature. The blood was centrifuged within 24 hours, and the plasma was withdrawn. To 1 bottle was added the 1.75 per cent globulin solution and to the other, the 3.50 per cent globulin solution. The globulin solution was cold. After thorough mixing, 3 equal aliquots of each were transferred to storage bottles. Each aliquot was transfused after storage for 1, 15, and 22 days respectively.

(27) α AND β GLOBULINS (FRACTION IV–3, 4, LOT 301)

Experiments Nos. 88, 90, 92, 94, 106, 108. (Controls 89, 91, 93, 105, 107, 109, 110 through 112 are described above.) Three grams of the globulin were dissolved in 100 ml. of the electrolyte solution. Two series of experiments were carried out, in one of which each test and control transfusion was a full bleeding, in the other of which each was a half-size bleeding. Resuspension was completed within 18 hours of drawing the blood. All solutions were cold and the packed cells were refrigerated.

Test transfusions were given 1, 11, 17, 24, 32, and 39 days, and control transfusions, 8, 10, 15, 18, 21, 22, 28, 31, and 38 days after bleeding.

The results of these experiments are summarized in Table V and Figure 3.

RESULTS

Whole blood

The poorest survival of whole blood was obtained with 4 per cent sodium citrate (No. 1). After 4 days of storage, 90 per cent of the cells were viable, but thereafter deterioration was precipitous.

All of the other solutions contained dextrose. The simplest of these, citrate and dextrose, was

TABLE V

Post-transfusion survival of human erythrocytes drawn as whole blood in 4 per cent sodium citrate and stored at 4° C. after resuspension in human serum protein-fortified electrolyte solutions

Solution	Transfusion no.	Days stored	Recip. blood group	Transfused volume: Total	Transfused volume: Cells	Age of tagged cells*	Clots	Reactions (febrile)	Survival
				ml.	*ml.*				*per cent*
5 per cent albumin + Hughes pH 6.8 (24)	36	10	0	67	33	27	++	+	91
	37	23	0	77	39	27	+++	+	54
Hughes pH 6.8	38	24	0	75	39	27	++	0	70
5 per cent albumin + Hughes pH 6.0 (25)	39	10	0	74	36	27	++	0	72
	40	23	0	71	35	27	++	0	38
Hughes pH 6.0	41	24	0	82	38	27	+++	+	30
Fraction IV–3, 4 (151) 1.75 per cent in Hughes (26)	82	1	0	167	79	132	+	0	82
	84	15	0	143	68	132	+	0	80
	86	22	A	142	68	132	+	0	78
Fraction IV–3, 4 (151) 3.5 per cent in Hughes (26)	83	1	0	166	77	141	+	0	95
	85	15	0	146	68	141	++	0	72
	87	22	A	141	67	141	+	0	65
Fraction IV–3, 4 (301) in Hughes (27)	88	1	A	505	212	181	0	0	98
	90	11	A	488	195	104	++	0	96
	92	17	A	525	215	104	++	0	90
	94	24	A	275	113	181	+	0	85
	106	32	0	198	90	104	+	0	53
	108	39	0	117	55	104	+	0	33

* Days between last injection of radioactive iron and day of bleeding.

De Gowin's solution (No. 3). Although there seemed to be some immediate cell damage, subsequent deterioration proceeded at a rate of less than 1 per cent a day up to about 20 days of storage, at which period 80 per cent of the cells proved to be viable. Thereafter, the survival curve broke sharply.

The 2 unmodified E.T.O. A and B solutions (Nos. 8 and 9) were fortified with dextrose, solution B also containing sodium chloride. Survival in both solutions was considerably better than in plain citrate, the 70 per cent levels being reached in about 11 and 15 days respectively.

Two solutions were buffered with phosphates, at 7.4 (McGill II) (No. 2), and at 7.1 (Parpart's) (No. 5). Survival in McGill II solution was about 90 per cent at 10 days of storage, but thereafter the rate of deterioration increased rapidly, the 70 per cent level coming on the sixteenth day. Very good preservation was obtained with Parpart's solution, even though some initial hemolysis may have been caused by taking the blood over dry citrate. During 26 days of storage, only about 10 per cent of the cells became nonviable.

The pH of Alsever's solution (No. 4) was adjusted to 6.8 with citric acid. Deterioration was fairly constant up to about 15 days of storage, when about 80 per cent of the cells were viable, the 70 per cent level being reached on the twentieth day.

Four solutions were adjusted to a pH of 5.0: E.T.O. Modifications A and B (Nos. 10 and 11) by hydrochloric acid, and ACD-1 (No. 6) and ACD-G (No. 7) by citric acid. Bloods in both of the modified E.T.O. solutions showed a constant rate of deterioration approximating 1 per cent per day, the 80 per cent level being reached in 20 and 16 days, respectively. This series was not followed down to the 70 per cent survival level. Both the ACD solutions gave very satisfactory preservation. Blood in ACD-1 deteriorated at less than 1 per cent per day for 25 days, and in ACD-G for 22 days, at which survival in both solutions was at least 80 per cent.

Deterioration of red cells resuspended in normal saline (No. 12) was rapid, the 90 per cent level being reached in 3 days, and practically no cells being viable after 10 days of storage.

Survival in the first series of cells in corn syrup (No. 13) was poor, the 70 per cent level being reached in about 4 days, but was considerably better in the second series. A wide scatter in survival was found in this series, in which the subjects were hospitalized patients, but it is evident that preservation was satisfactory for at least 10 days.

Both the phosphate-buffered solutions (Nos. 14 and 15) gave good preservation.

Surivival of cells in the pH 6.8 solution was better than 80 per cent up to the longest storage period studied, 20 days; and the survival of cells in the pH 6.0 solution was 70 per cent at 22 days.

The citrate-buffered solution adjusted to pH 3.8 with HCl (No. 27, controls[10]) was better than that adjusted to pH 5.1 (No. 16), the 70 per cent survival levels being reached in 20 and 14 days respectively.

Two other citrate-buffered solutions, C_2A (No. 18) and C_2A^1 (No. 19), gave fairly good preservation, the 70 per cent levels being reached at 21 and 18 days respectively.

The low volume citrate-buffered solution (No. 20) gave poor preservation, the survival level having fallen to about 10 days, and to less than 60 per cent after 16 days of storage.

Survival of cells in the bicarbonate-buffered solution (No. 21) was 70 per cent after 20 days but only about 40 per cent after 26 days of storage.

Packed red cells

Red cells drawn into ACD-G, stored as centrifuged cells with the plasma removed, and transfused up to 21 days of storage without the addition of any diluent, survived as well as whole blood in the same solution. The addition of 0.85 per cent NaCl; 1.1 per cent, 0.85 per cent and 0.6 per cent NaCl plus 0.5 per cent of dextrose; and of 0.85 per cent NaCl plus 0.5 per cent dextrose and 0.2 per cent citric acid decreased the viability of the cells as compared to whole blood, the 70 per cent level being reached at from 12 to 18 days.

The addition just prior to transfusion of 1.0 per cent NaCl; of 1.0 per cent NaCl plus 0.5 per cent dextrose; and of 0.85 per cent NaCl plus 0.5 per cent dextrose did not increase the viability of the cells as compared with whole blood. Results with the addition of 10 per cent corn syrup were equivocal.

[10] Illustrated in Figure 3.

Solutions with added human serum protein fractions.

Human serum albumin (Nos. 24 and 25) did not increase the preservative quality of the electrolyte solution in which it was added (Nos. 14 and 15). Fraction IV–3, 4, composed of α and β globulins and albumin, apparently had a beneficial effect. With one lot of globulin (No. 26) survival of better than 70 per cent was obtained for 15 days. With the other lot (No. 27) survival of cells in the globulin-containing solution was better than 80 per cent for 24 days of storage, but survival of the cells in the control solution had fallen to 71 per cent over a similar storage period.

DISCUSSION

Most of the experimental subjects were normal ambulatory males. In the series of cells resuspended in corn syrup (Experiments Nos. 140 through 147), and Hughes pH 5.1 solution (Experiments Nos. 132 through 139), hospitalized patients were used. It will be noted in Figure 2 that the scatter in percentage of survival throughout the period of observation (9 to 20 days) was much greater in both series than that seen in the normal recipients. Most of these patients were anemic, some were mildly febrile, some had gastrointestinal lesions, others were postoperative, and one under daily X-ray therapy for carcinomatosis. Similar observations on survival of radioactively tagged stored red cells are reported by Denstedt (47) in mental cases and by Strumia (48) in hospital ward patients. Accordingly, the survival in normals may be regarded as the best experimental basis for the comparative evaluation of viability of blood stored in various solutions.

The size of transfusions given varied from full bleedings (150 to 250 ml. of cells) to small aliquots (25 to 100 ml. of cells). Thus, the amounts transfused represented variable percentages of the recipient's pre-transfusion red cell volume. Since the recipients' red cell volumes ranged from about 1,800 to 2,500 ml., the transfusions ranged from 1 to 15 per cent of total red cell volume. The time in which the transfusion was given, and hence the average rate of flow, also varied.

The absolute quantities of non-viable cells varied, in these experiments, with the size of the transfusion and the percentage of survival. While these factors might well influence the *rate* at which non-viable cells or hemoglobin were removed from circulation, it is hardly to be expected that many non-viable cells, even in a badly deteriorated blood, would remain in active circulation for any considerable period of time. Thus the accuracy with which the proportion of non-viable to viable transfused cells was measured was not affected by the size of the transfusion.

In the paper in which the radioactive iron method of measuring post-transfusion survival of transfused red cells (40) was described, the percentage of survival, that is, the percentage of transfused cells found viable, was based upon the lowest radioactivity value of the recipient's red cells found during the observation period. This low point occurred within the first 24 hours after transfusion in the majority of cases, and seldom beyond the first 48 hours after transfusion.

Recipient radioactivity levels after 48 hours constitute the sum of radioactivity of retained donor cells and of new cells containing re-utilized radio-iron. Thus the method does not measure the continued longest period of survival of viable donor cells in the recipient's blood stream. However, the chief danger from the transfusion of stored blood lies in the extent of breakdown of donor cells, and hence the *immediate* survival of the transfused cells becomes a factor of paramount importance in evaluating preservative solutions.

The limit of error of the technic was defined as ± 5 per cent. In those cases in which the loss of cells was considerable, it is possible that some re-utilization of liberated radio-iron had already occurred at the time that the lowest recipient radioactivity value was obtained. If such were the case, the extreme loss of transfused cells might not have been detected, and the computed survival may have been falsely high. In all probability this potential error is not significant in the results obtained on bloods showing better than 60 per cent survival.

It should be clearly understood that only radioactively tagged erythrocytes are detected by the analytic procedures employed. The exact percentage of tagged cells of given ages in the blood of any donor cannot be calculated from the data available, since the quantity of the radio-iron in the individual corpuscles was not measured, nor is it certain that radio-iron is evenly distributed in all developing cells. The time elapsed between the

first exposure of a donor to radio-iron and the drawing of blood from that donor is known and is listed for each transfusion in Tables II through V. Thus it is possible to consider donors as having predominantly young, middle-aged, or old tagged cells. Those donors in whom the time interval between administration of radio-iron and drawing of blood exceeded the normal life span of the red cell (about 100 days) may be considered as having, to an unknown extent, a mixed-age cell population. The longer the time interval, the more closely would the age distribution of the tagged cells approach the age distribution of all the circulating cells.

It was also stated (40) that the age of the radioactively tagged cells at the time the blood was drawn was a factor in the rate of deterioration of the cells during storage; that very young cells survived about 10 per cent better during storage than did cells of advanced age. In those experiments in which only young tagged cells were given, the survival percentages might therefore be expected to be somewhat better than if tagged cells of ages more nearly representative of the whole red cell population had ben used.

Thus, the survival of tagged cells from any donor could be expected to be best when all cells are young and to become progressively poorer as they approach the end of their life span. After that time, survival would be improved due to the presence of new cells tagged with re-utilized radio-iron. This phenomenon would continue until all ages of cells were represented by tagged cells, and the measured survival of the radioactive cells would be truly representative of the survival of the blood as a whole.

The maximum differential in survival of young and old cells is 10 per cent, and the degree of this error will tend to diminish the more closely the age distribution of the tagged cells approaches that of a truly mixed-age red cell population. This factor, slight as it is, has been taken into consideration in the final evaluation of the preservative solutions studied.

In general, evidence, by the radioactive method, of good preservation is slightly more significant when the transfusion contained predominantly old or mixed-age tagged cells, than when the tagged cells were young. Conversely, evidence of poor preservation is more significant when the transfusion contained young tagged cells, than when the tagged cells were of mixed or advanced age.

Studies by Shemin and Rittenberg, employing N^{15}, have demonstrated that the normal life span of human erythrocytes is from 100 to 120 days (33). It can therefore be assumed that red cells normally die off at the rate of about one per cent a day in the body. It follows that if cells deteriorate at a similar rate during storage, as determined by post-transfusion survival, preservation *in vitro* has been as good as if they had remained in circulation in the body.

Survival at a rate equivalent to the post-transfusion loss of not more than 1 per cent of the transfused cells per day of storage would therefore seem a reasonable standard for "satisfactory" preservation. The slope of this rate of survival is plotted in broken lines in Figures 1, 2, 3.

This does not imply that blood showing higher rates of deterioration cannot be safely transfused. As will be shown in a subsequent communication (49), the normal reticulo-endothelial and erythropoietic system is capable of disposing of red cell breakdown products up to 30 per cent of a 500 ml. transfusion without marked hemoglobinemia or hemoglobinuria. One important factor is, therefore, the storage period at which not less than 70 per cent of transfused cells survive. With what we have defined above as "satisfactory" preservation, survival would decline to this level in 30 days. This "zone of safe transfusion" is indicated by the dotted rectangle in Figures 1 through 3.

Inspection of the time-survival curves in these figures reveals that the initial rate of deterioration remains fairly constant throughout the observation period, but in others becomes accelerated at some point during continued storage.

This constant rate of deterioration is illustrated in Figure 1 by the time-survival curves of Parpart's solution, ACD–1 and ACD–G, and E.T.O. Modification A. The acceleration in rate of deterioration is illustrated by the time-survival curves in Figure 1 of citrated blood, McGill II, De Gowin's and Alsever's solution, and in Figure 3, by the curves of cells resuspended in the globulin-fortified (301) acid-citrate dextrose solution, and the pH 3.8 controls. The preservative showing a low and constant rate of deterioration over substantial periods would appear to be superior to one in which an abrupt change was observed.

Both of those characteristics must be considered in determining the longest permissible dating period of blood stored in any preservative.

The storage period at which the rate of deterioration began to exceed 1 per cent per day, and at which the smoothed time survival curves intersect the 70 per cent survival abscissa, is shown in Table VI for each solution studied. In those series in which survival did not fall below 70 per cent, the percentage of survival obtained on the longest observed storage period is listed. Also given in the table are "dating periods"—the longest permissible safe periods of refrigerated storage.

Any dating period is at best an approximation. Blood from individual donors presents one variable, and the state of health of recipients many more variables. The manner in which blood is drawn, and its subsequent handling also may affect behavior during storage. The determination of upper limits of storage should be based on conservative rather than optimistic considerations.

It is recognized that the ability of the human body to dispose of red cell breakdown products is extremely variable and may be far greater than hitherto believed. In the opinion of the authors, safe practice would appear to be to give the patient, not the preservative solution, the benefit of the doubt.

These values have been arrived at by considering the characteristics of the time-survival curves: the storage period at which the rate of deterioration began to exceed 1 per cent per day and the survival had dropped off to 70 per cent (where observed). The age of the tagged cells was also considered according to the general statement made above.

There are obviously wide differences in the preservative qualities of the solutions studied. They may be grouped according to dating period somewhat as shown in Table VII.

In light of the data presented, it can be empirically stated that maximal preservation of cells stored as whole blood is obtained with a solution in which

(a) the ratio of whole blood to diluent is not less than 4 to 1;

(b) the concentration of citrate in diluted plasma is between 0.4 and 0.6 grams per 100 ml. (about 1.0 gram per 100 ml. of packed cells);

TABLE VI

Storage periods at which rate of deterioration begins to exceed 1 per cent per day, at which the 70 per cent survival level is reached, and approximate "dating periods"

Solution	Sol. no.	Storage period at which: Rate of deterioration >1 per cent per day	70 per cent survival level reached	"Dating period"
Whole blood				
Sodium citrate	1	3	6	5
McGill solution	2	11	14	12
DeGowin's	3	19	24	20
Alsever's	4	16	20	18
Parpart's	5	+27	+27	26
ACD-1	6	27	29	26
ACD-G	7	22	+22	22
E.T.O. citrate	8	5	10	7
E.T.O. citrate-saline	9	?*	14	10
E.T.O. Mod. A	10	20	+20	20
E.T.O. Mod. B	11	?*	+21	20
Cells resuspended in electrolyte solutions				
0.85 per cent saline	12	0	6	4
10 per cent corn syrup**	13	10	13	10
Phosphate-buffered citrate-dextrose pH 6.8	14	+20	+20	20
Phosphate-buffered citrate-dextrose pH 6.0	15	?*	23	20
Citrate-buffered citrate-dextrose pH 5.0	16	10	13	10
Citrate-buffered citrate-dextrose pH 3.8	17	19	24	20
Modified ACD C_2A	18	11	19	14
Modified ACD C_2A^1	19	16	22	15
Modified ACD (Washington Conference)	20	6	13	9
Bicarbonate-buffered citrate-dextrose	21	14	21	16
Cells resuspended in protein-fortified solutions				
Phosphate-buffered citrate-dextrose plus albumin pH 6.8	24	11	17	12
Phosphate-buffered citrate-dextrose plus albumin pH 6.0	25	?	11	8
Citrate-buffered citrate-dextrose plus globulin pH 7.0	26	?	+22	20
Citrate-buffered citrate-dextrose plus globulin pH 3.8	27	+23	28	24
Centrifuged cells with and without added saline				
ACD-1 with saline	22	12	18	14
ACD-G with saline	23	10	19	14
ACD-G without saline		+21	+21	20

* Observations not made early enough in storage period to determine initial rate of deterioration.
** Second series, Experiments Nos. 140 through 147.

(c) the concentration of dextrose in the diluted plasma after equilibration with the cells is about 0.5 gram per 100 ml.; and

(d) the hydrogen ion concentration is high

TABLE VII

Upper safe limits of storage periods for whole blood and cell resuspension solutions

Days of storage				
0–5	5–10	10–15	15–20	20–25
Whole blood				
Sodium citrate	E.T.O. citrate E.T.O. citrate saline	McGill	DeGowin's Alsever's E.T.O. Mod. A E.T.O. Mod. B	Parpart's ACD-1 ACD-G
Resuspended cells—electrolytes				
0.85 per cent NaCl	10 per cent corn syrup Citrate-buffered citrate-dextrose pH 5.0 Modified ACD (Wash. Conf.)	Modified ACD C_2A C_2A^1	Phosphate-buffered citrate-dextrose pH 6.8 pH 6.0 Citrate-buffered citrate-dextrose pH 3.8 Bicarbonate-buffered citrate-dextrose	
Resuspended cells—protein-fortified solutions				
	Phosphate-buffered citrate-dextrose plus albumin			Citrate-buffered citrate-dextrose plus globulin
		ACD-1 in saline* ACD-G in saline	ACD-G without saline	

* Added just prior to transfusion.

enough so that the buffering action of plasma proteins and hemoglobin will not raise the pH of the diluted plasma above 7.0, or of the cells above 6.8.

These conclusions are in keeping with those of Mollison (35), Loutit (36), and Altmann and Watson (50).

The specifications of the optimal resuspension fluid are, however, less clear.

Since the cells were drawn as whole blood into 4 per cent sodium citrate, some initial damage may have occurred before resuspension.

The addition of dextrose and the maintenance of a slight acidity of the diluent *during storage* appear to be as important for resuspended cells as for whole blood. No significant hemolysis was produced by the addition of even the most acid fluids.

These observations do not apply to fluids used for resuspension of cells drawn as whole blood into ACD-1 or ACD-G.

Slightly hypertonic solutions of NaCl with or without low concentrations of dextrose can be safely used for diluting packed ACD red cells provided the diluent is added just prior to transfusions. No electrolyte diluent was found in which packed cells could be stored without increasing their rate of deterioration.

The formation of fibrin clots occurred in more or less degree in all stored blood and resuspended cells. They varied in character from very fine, pale particles to larger, tenacious, gelatinous masses. They were roughly quantitated by inspection of filters and bottles at the end of transfusion. The relative amounts of clot noted are listed in Tables I to IV. Those clots quantitated as 1 + or 2 + did not interfere with administration of blood through a cylindrical, metal, 100-mesh screen filter with a 20 sq. cm. surface area. Those indicated as 3 + were in sufficient quantity to slow down the speed of transfusion but not stop flow. The small particles cause obstruction by coating

the filter surface and are of more significance than the large, stringy masses which might possibly block the outlet from the bottle, although this has not occurred in our experience.

The amount of clot in any given solution tended, in general, to increase on storage at optimal temperature, the oldest bloods showing the largest amounts. The degree of clot formation on storage showed no relationship to ratio of volume of whole blood to anticoagulant used. There was generally less clot present in stored cell resuspensions than in stored whole blood. Albumin solution and saline resuspensions were notable exceptions, both showing relatively large amounts of clot of the stringy, gelatinous type. In stored packed ACD-1 and ACD-G cells, there was much less clot formation than in the same solutions stored as whole blood. None of the solutions studied entirely prevented the formation of clots.

The reactions tabulated in Tables II to V refer only to those of the febrile type and were probably due to accidental pyrogenic contamination of some part of the collecting, resuspending or transfusion apparatus, or of the solution used, rather than to the breakdown of cells. In many instances there was donor cell destruction equivalent to or greater than that occurring in subjects having a rise in temperature, with no symptoms or fever. In no instance of febrile reaction was bacterial contamination demonstrated. In every case where there was a rise in temperature, there were also malaise and other related symptoms.

In 52 transfusions of whole blood, there were 3 very mild reactions with a maximum temperature rise of 1.5° F., with no chill, and 1 with a maximum rise of 3.1° F., with a slight chill. In 81 transfusions of resuspended or packed cells, there were 7 very mild reactions, with a temperature rise of less than 1.0° F., 5 with a 1.0 to 1.5° F. temperature rise; and 2 with a temperature rise of 3.1 and 4.4° F., respectively, and these 2 subjects experienced chills.

There were about twice as many reactions in bloods stored 15 days or less than in those stored more than 15 days. Hence prolonged storage did not increase the incidence of pyrogen reactions.

It is felt that the information obtained from this admittedly small series of well controlled experimental transfusions offers a better basis for evaluating preservatives than observations obtained by less accurate clinical laboratory tests in larger series of observations.

The practical worth of any preservative must finally rest on long-range clinical experience. The conclusions reached herein are set forth as a guide to clinical usage: for the selection of solutions to meet specific requirements and for the establishment of safe storage limits.

CONCLUSIONS

(*1*) The post-transfusion survival of preserved human erythrocytes, stored under refrigeration for varying periods of time, has been determined by means of the radioactive iron technic.

(*2*) Studies were made of 11 preservative solutions for whole blood, of 10 electrolyte solutions for resuspensions of centrifuged cells, of 2 electrolyte resuspension fluids to which human serum protein fractions were added, and of packed cells stored without resuspension after the removal of plasma.

(*3*) The longest period of time at which cells stored under refrigeration can be safely transfused has been determined for every solution investigated. This "dating period" is based upon the observed rate of deterioration of the red cells during storage.

(*4*) Refrigeration, the addition of dextrose to the citrate anticoagulant, the maintenance of a slightly acid reaction of the diluted plasma or resuspension fluid, and optimal dilution are essentials for prolonged preservation.

(*5*) Whole blood can be preserved up to 70 per cent viability for at least 21 days in acid-citrate (ACD-1 and ACD-G), and for at least 15 days in De Gowin's, Alsever's, and McGill solutions.

(*6*) Red cells drawn as whole blood into sodium citrate can be preserved by resuspension in citrate-buffered citrate-dextrose solution for from 10 to 15 days.

(*7*) Packed red cells from blood drawn into acid-citrate-dextrose may be safely transfused both without the addition of diluent, and with a slightly hypertonic saline diluent added just prior to transfusion, up to 21 days of storage.

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BIBLIOGRAPHY

1. Shattock, S. G., Chromocyte clumping in acute pneumonia and certain other diseases, and the significance of the buffy coat in the shed blood. J. Path. & Bact., 1900, 6, 303.
2. Landsteiner, K., Über Agglutinationserscheinungen normalen menschlichen Blutes. Wein. Klin. Wchnschr., 1901, 14, 1132.
3. Jansky, J., Haematologické, studie u psychotiků. Sborn. klin., 1906–07, 8, 85.
4. Moss, W. L., Studies on isoagglutinins and isohemolysins. Johns Hopkins Hosp. Bull., 1910, 21, 63.
5. Kimpton, A. R., and Brown, J. H., A new and simple method of transfusion. J. A. M. A., 1913, 61, 117.
6. Lindeman, E., Simple syringe transfusion with special cannulas; a new method applicable to infants and adults; preliminary report. Am. J. Dis. Child., 1913, 6, 28.
7. Unger, L. J., A new method of syringe transfusion. J.A.M.A., 1915, 64, 582.
8. Agote, L. Nuevo procedimento para la transfusion de la sangre. Rev. med.-quir., 1920, 1, 3.
9. Lewisohn, R., A new and greatly simplified method of blood transfusion. A preliminary report. Med. Rec., 1915, 87, 141.
10. Hoffman, M. H., and Habein, H. C., Transfusion of citrated blood. J. A. M. A., 1921, 76, 358.
11. Brines, O. A., The transfusing of unmodified blood. Arch. Surg., 1923, 7, 306.
12. Herr, E. A., Blood transfusion to date. Surg., Gynec., & Obst., 1925, 41, 513.
13. Rous, P., and Turner, J. R., The preservation of living red blood cells *in vitro*. J. Exper. Med., 1916, 23, 219.
14. Robertson, O. H., Transfusion with preserved red blood cells. Brit. M. J., 1918, 1, 691.
15. Levine, P., and Katzin, E. M., A survey of blood transfusion in America. J. A. M. A., 1938, 110, 1243.
16. Wechselmann, Neuere erfahrungen über intravenöse salvarsaninjektionen ohne reaktionserscheinungen. München Med. Wchnschr., 1911, 58, 1510.
17. Seibert, F. B., Fever-producing substance found in some distilled waters. Am. J. Physiol., 1923, 67, 90.
18. Banks, H. M., Study of hyperpyrexia reaction following intravenous therapy. Am. J. Clin. Path., 1934, 4, 260.
19. Co Tui, McCloskey, K. L., Schrift, M., and Yates, A. L., New method of preparing infusion fluids, based on removal of pyrogen by adsorptive filtration. J. A. M. A., 1937, 109, 250.
20. Lees, J. C., and Levvy, G. A., Emergency preparation of pyrogen-free water. Brit. M. J., 1940, 1, 430.
21. Walter, C. W., Preparation of safe intravenous solutions. Surg., Gynec., & Obst., 1936, 63, 643.
22. Walter, C. W., Economical intravenous therapy. J. A. M. A., 1935, 104, 1688.
23. Davidsohn, I., Isoagglutinin titers in serum disease, in leukemias, in infectious mononucleosis, and after blood transfusions. Am. J. Clin. Path., 1938, 8, 179.
24. Landsteiner, K., and Wiener, A. S., An agglutinable factor in human blood recognized by immune sera for rhesus blood. Proc. Soc. Exper. Biol. & Med., 1940, 43, 223.
25. Fantus, B., Therapy of Cook County Hospital; blood preservation. J. A. M. A., 1937, 109, 128.
26. Lundy, J. S., and Osterberg, A. E., Intravenous administration of fluids including blood transfusion. Surg., 1937, 2, 590.
27. Gwynn, C. A., and Alsever, J. B., The collection and preservation of placental blood for transfusion purposes. Am. J. M. Sc., 1939, 198, 634.
28. De Gowin, E. L., Harris, J. E., and Plass, E. D., Studies on preserved human blood. I. Various factors influencing hemolysis. J. A. M. A., 1940, 114, 850.
29. Idem, II. Diffusion of potassium from the erythrocytes during storage. J. A. M. A., 1940, 114, 855.
30. De Gowin, E. L., Harris, J. E., Bell, J., and Hardin, R. C., Osmotic changes in erythrocytes of human blood during storage. Proc. Soc. Exper. Biol. & Med., 1942, 49, 484.
31. De Gowin, E. L., Hardin, R. C., and Swanson, L. W., Studies on preserved human blood. IV. Transfusion of cold blood into man. J. A. M. A., 1940, 114, 859.
32. De Gowin, E. L., and Hardin, R. C., Studies on preserved human blood. VI. Reactions from transfusion. J. A. M. A., 1940, 115, 895.
33. Shemin, D., and Rittenberg, D., Studies on the formation of heme and on the average life time of the human red cell. Federation Proceedings, 1946,, Vol. 5, Part II, p. 153.
34. Wiener, A. S., and Schaefer, G., Limitations in the use of preserved blood for transfusion. A study of the fate of the transfused erythrocytes in the recipient's circulation. Med. Clin. N. Amer., 1940, 24, 705.
35. Mollison, P. L., and Young, I. M., Survival of the transfused erythrocytes of stored blood. Lancet, 1940, 2, 420.
36. Loutit, J. F., Mollison, P. L., and Young, I. M., Citric acid-sodium citrate-glucose mixtures for blood storage; report to Medical Research Council from Southwest London Blood Supply Depot. Quart. J. Exper. Physiol., 1943, 32, 183.
37. De Gowin, E. L., The storage, transportation and administration of whole blood. Blood Substitutes and Blood Transfusion. Charles C. Thomas, Springfield, Ill., 1942, pp. 277–296.

38. Peacock, W. C., Evans, R. D., Irvine, J. W., Jr., Good, W. M., Kip, A. F., Weiss, S., and Gibson, J. G., 2nd, The use of two radioactive isotopes of iron in tracer studies of erythrocytes. J. Clin. Invest., 1946, 25, 605.
39. Gibson, J. G., 2nd, Weiss, S., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., Good, W. M., and Kip, A. F., The measurement of the circulating red cell volume by means of two radioactive isotopes of iron. J. Clin. Invest., 1946, 25, 616.
40. Gibson, J. G., 2nd, Aub, J. C., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., and Sack, T., The measurement of post-transfusion survival of preserved stored human erythrocytes by means of two isotopes of radioactive iron. J. Clin. Invest., 1947, 26, 704.
41. Gibson, J. G., 2nd, and Evelyn, K. A., Clinical studies of the blood volume. IV. Adaptation of the method to the photoelectric microcolorimeter. J. Clin. Invest., 1938, 17, 153.
42. Denstedt, O. F., Osborne, D. E., Stansfield, H., and Rocklin, I., The survival of preserved red cells after transfusion. Anesthesiology, 1944, 5, 237.
43. De Gowin, E. L., Harris, J. E., and Plass, E. D., Changes in human blood preserved for transfusion. Proc. Soc. Exper. Biol. & Med., 1939, 40, 126.
44. Parpart, A. K., Personal communication.
45. Rapoport, S., Personal communication.
46. Thalhimer, W., and Taylor, E. S., The transfusion of centrifuged type O cells, resuspended and stored in 10 per cent corn syrup. J. A. M. A., 1945, 127, 1096.
47. Denstedt, O. F., Personal communication.
48. Strumia, M. M., Personal communication.
49. Gibson, J. G., 2nd, Peacock, W. C., Evans, R. D., Sack, T., and Aub, J. C., The rate of post-transfusion loss of non-viable stored human erythrocytes and the re-utilization of hemoglobin-derived radioactive iron. J. Clin. Invest., 1947, 26, 739.
50. Altmann, A., and Watson, E. F., Survival of transfused erythrocytes of stored blood. South African M. J., 1945, 19, 167.

THE RATE OF POST-TRANSFUSION LOSS OF NON-VIABLE STORED HUMAN ERYTHROCYTES AND THE RE-UTILIZATION OF HEMOGLOBIN-DERIVED RADIO-ACTIVE IRON [1]

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When human erythrocytes are transfused within a few hours of taking in citrate solution, practically all of them remain intact in the recipient's blood stream (1). Thereafter, they disappear from the circulation at a rate of about one per cent per day (2, 3), and it is generally believed that this is the normal death rate of homologous erythrocytes.

Chemico-physical changes occur in red cells during storage *in vitro*. Depending upon the preservative value of the anticoagulant used, deteriorative changes may occur gradually and proceed at relatively constant rates for considerable periods, or they may be initiated early in storage and proceed at accelerated rates. Post-transfusion viability of preserved cells is a function of the length of storage (1).

Thus, the transfusion of stored blood, which always contains a certain quantity of cells in which irreversible changes have occurred, necessitates the removal of those dead or dying red cells from the circulation. These cells may rupture in the blood stream and, through release of hemoglobin, give rise to hemoglobinemia. It is also possible that they may be removed, with membranes relatively intact, by phagocytosis, or segregation in the spleen (4), and subsequently destroyed. In either event a load is imposed upon the mechanism for scavenging the blood stream and for the handling of hemoglobin-derived pigments: the reticulo-endothelial and the erythropoietic systems. Hemoglobinemia also involves renal function since there is a plasma level for hemoglobin above which hemoglobinuria occurs. The degree of this burden will be determined by the quantity of mortally damaged cells transfused (which will in turn depend upon the size of the transfusion, and, for any given preservative solution, the length and conditions of storage prior to transfusion), and the rate at which the blood is transfused.

In previous communications (5) it was concluded that the greater part of the non-viable cells are completely removed from active circulation, usually within 24 hours after transfusion, and that the remainder of the transfused cells resume normal functional capacity and enjoy a sojourn in the body equivalent to the remainder of their normal life expectancy. It is therefore only the non-viable portion of the transfused red cells that need be considered in the study of the effects of the transfusion of stored blood.

In a number of experiments in which blood, the cells of which were tagged with radioactive iron, was transfused, the initial recipient blood sample was taken within 60 minutes, and several subsequent samples within the first 14 hours of the beginning of the transfusion. Since little if any re-utilization of hemoglobin-derived radio-iron can be expected to occur in this period, the radioactivity of those blood samples accurately measured the quantity of transfused cells removed from the blood stream. These values may be expressed in terms of the percentage of total cells transfused or of the contained quantities of hemoglobin or of iron.

Such data were obtained in 9 transfusions of stored whole blood and in 15 transfusions of stored resuspended cells. The whole bloods were drawn into ACD-1 and Alsever's solution; they were transfused from 8 to 26 days after collecting.

Five of the bloods in ACD-1 had been transported, by

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts Institute of Technology, in collaboration with the Peter Bent Brigham Hospital, and the Massachusetts General Hospital.

TABLE I

Quantities of whole blood (or resuspensions) and red cells transfused, storage periods, and percentages of non-viable red cells removed from circulation at successive intervals from beginning of transfusion

Exp. no.	Solution	Days stored	Transfused: Total	Transfused: Cells	Time intervals in minutes from start of transfusion / Per cent cells removed in interval				
			ml.	ml.					
69	ACD-1	8	543	247	35 5	88 6	233 6	428 7	1,320 9
72	ACD-1	15	536	230	95 4	147 5	322 7	1,320 14	
73	ACD-1	16	565	253	58 2	114 3	354 7	1,470 8	
76	ACD-1	25	325	150	36 0	99 7	249 22	449 26	1,290 30
77	ACD-1	26	285	121	36 1	112 2	232 6	1,350 17	
78	Alsever's	19	575	161	50 16	117 33	242 55	482 67	1,260 76
79	ACD-1	20	366	156	54 28	91 44	236 72	1,260 83	
80	Alsever's	23	515	151	38 35	95 57	476 67	1,260 87	
81	ACD-1	24	285	122	34 22	89 55	239 82	479 82	1,290 92
88		1	505	212	57 0	127 0	337 0	1,320 5	
90	Citrate-buffered citrate-dextrose with added globulin	18	420	191	52 0	117 1	363 3	1,260 6	
92		17	525	215	55 0	106 5	246 7	1,260 10	
133		13	467	217	65 6	155 12	355 28	1,440 40	
134		15	520	235	153 5	372 28	1,500 29		
135		20	315	147	43 11	123 32	273 36	1,615 47	
136	Citrate-buffered citrate-dextrose pH 5.0	11	510	256	55 11	135 32	335 36	1,500 47	
137		14	500	234	46 13	130 28	290 45	1,440 52	
138		15	497	245	55 22	124 34	392 58	1,500 68	
139		18	357	175	76 16	176 26	326 42	1,410 45	
140		13	502	227	61 —	164 3	349 6	1,500 8	
141		9	487	169	45 2	140 4	300 6	1,530 11	
142	10 per cent corn syrup	15	475	178	44 14	74 21	184 43	1,440 46	
143		20	470	193	61 28	115 —	275 33	1,440 59	
146		14	520	220	75	155	350	1,360	

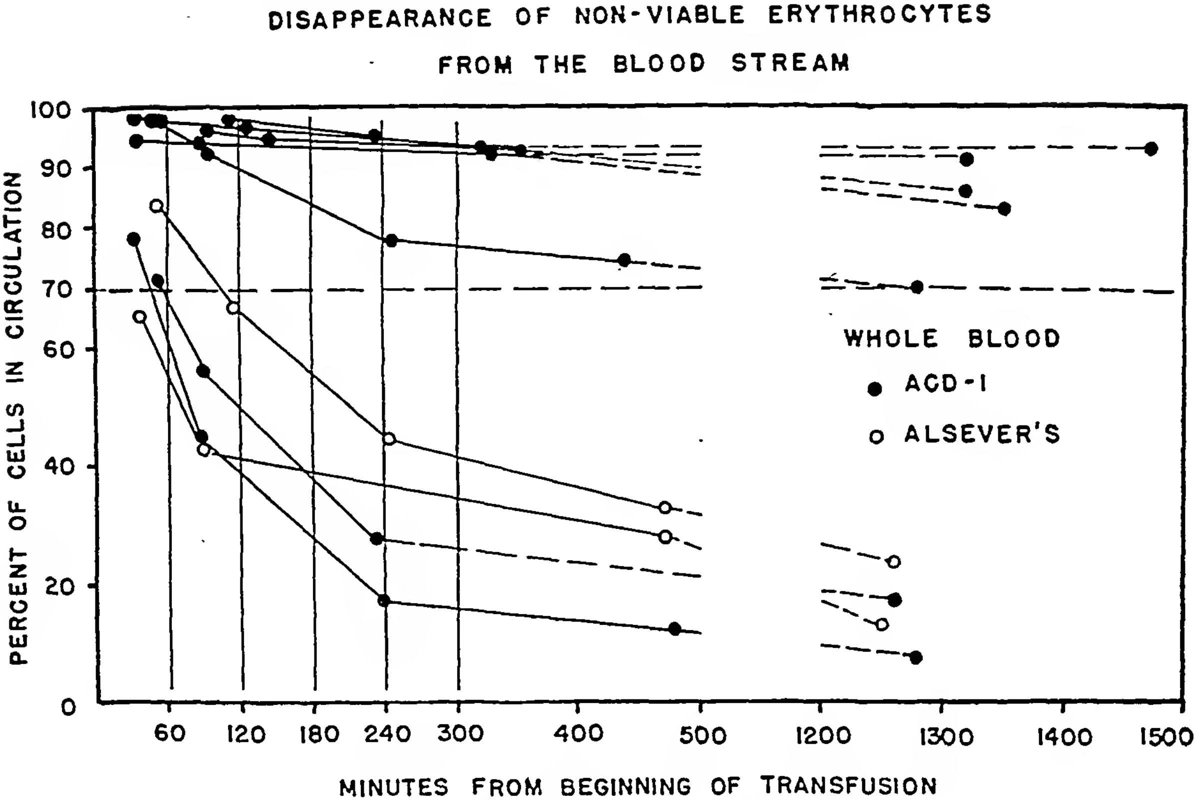

FIG. 1. DISAPPEARANCE OF NON-VIABLE ERYTHROCYTES FROM THE BLOOD STREAM

The percentage of total transfused tagged cells stored as whole blood in ACD-1 and Alsever's solution remaining in circulation during the first 1,500 minutes after beginning transfusion.

air, under constant refrigeration, from Boston, Massachusetts to Oakland, California, and back (5 days elapsed), and subsequently were stored at 4° C. Two bloods in ACD-1 and 3 in Alsever's solution had been transported by air, without constant refrigeration, from Boston to Paris, France (11 days elapsed), and subsequently were stored at 4° C.

The packed cells were resuspended in: a 10 per cent corn syrup; a citrate-buffered citrate-dextrose solution of pH 5.0; and a citrate-buffered citrate-dextrose solution containing 3 per cent Fraction IV-3, 4, Lot 301[2] (α and β globulin). The cells in the globulin-fortified solution had been in depot storage at 4° C. until transfused. The cells in corn syrup and in the acidified-citrate-dextrose solution were transported by truck, under controlled refrigeration (4 to 10° C.), from Boston to New York, and transfused into patients on the wards of the New York Hospital.[3]

[2] Prepared in the Pilot Plant of the Department of Physical Chemistry, Harvard Medical School, under the direction of Dr. E. J. Cohn.

[3] These experiments were carried out, under request from medical authorities of the U. S. Navy, represented by Lt. (s.g.) Henry Blake, U.S.N.R., in collaboration with Dr. Ralph G. Stillman, of the New York Hospital, and Dr. William Thalhimer.

The transfused quantities of whole blood in ACD and Alsever's solution ranged from 315 to 525 ml. (121 to 253 ml. of cells); cell resuspensions in corn syrup and in the citrate-buffered citrate-dextrose ranged from 315 to 520 ml. (147 to 256 ml. of cells); and cell resuspensions in the globulin-fortified resuspension solution, from 420 to 505 ml. (171 to 215 ml. of cells). The average of the 24 transfusions was 194 ml. Time required for transfusion varied from 13 to 65 minutes, and the rate of flow from 8 ml. to 36 ml. per minute, averaging 18 ml. per minute. The hematocrits of whole blood were from 28 to 46; and of cell resuspensions from 34.7 to 50.5; the average hematocrit of all the transfusions was 42.7. Survival during the first 24 post-transfusion hours ranged from 92 to 8 per cent for whole bloods and from 95 to 32 per cent for the cell resuspensions.

These quantities of whole blood and cells are well within the ranges encountered in single clinical transfusions; hence the data may be treated as a whole in applying the findings to transfusion practice. The average rate of inflow, 18 ml. per minute, is perhaps more rapid than that of the usual routine transfusion.

The quantity of whole blood or resuspension cells transfused, rate of inflow, and percentage of total transfused cells lost from circulation at suc-

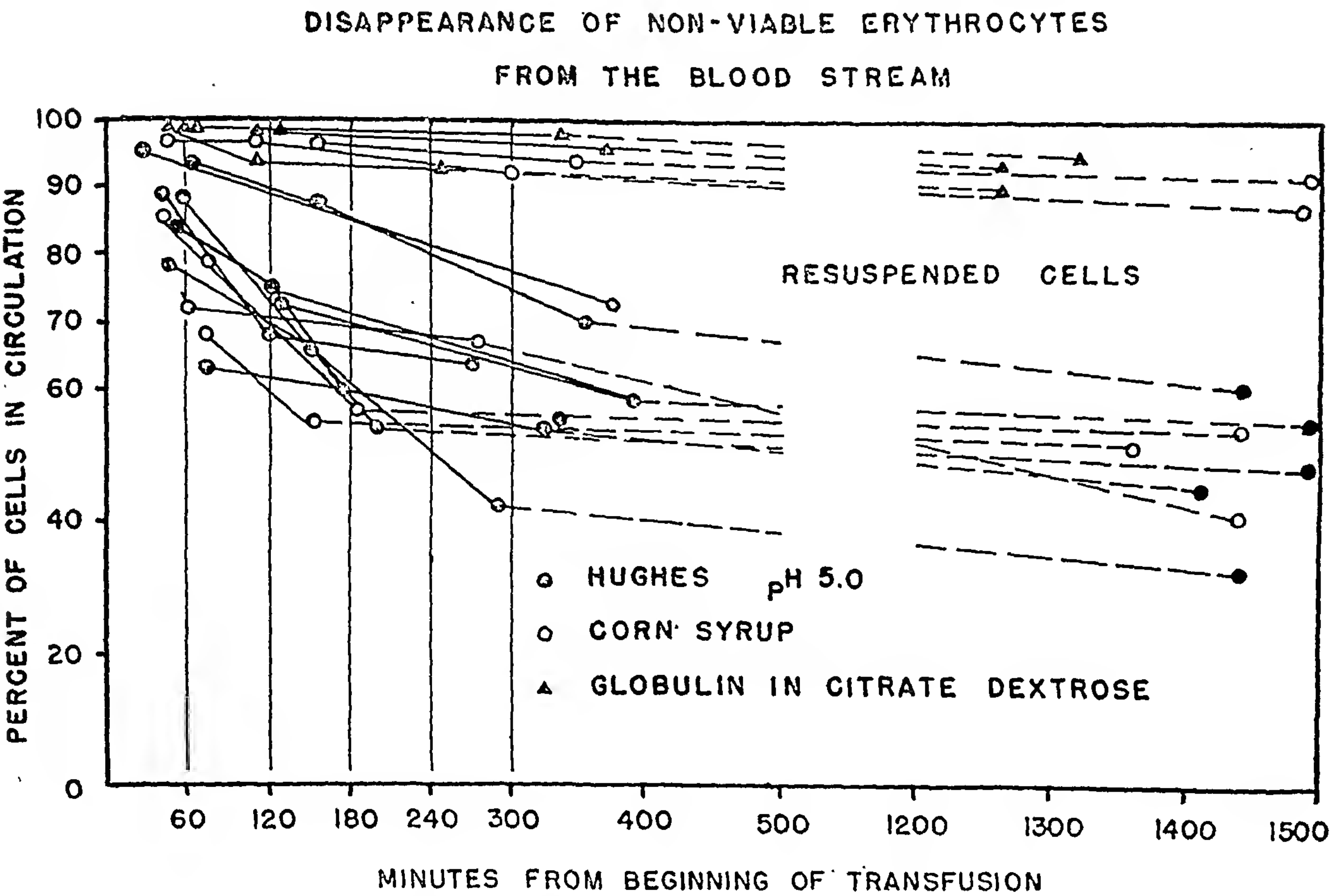

FIG. 2. DISAPPEARANCE OF NON-VIABLE ERYTHROCYTES FROM THE BLOOD STREAM

The percentage of total transfused tagged cells drawn as whole blood in 4 per cent sodium citrate and resuspended in 3 preservative solutions, remaining in circulation during the first 1,500 minutes after beginning transfusion.

cessive intervals from the beginning of the transfusion are shown in Table I. The percentages of cells remaining in circulation at intervals up to 1,500 minutes after transfusion are shown in Figures 1 and 2.

The post-transfusion behavior of both the cells stored as whole blood and the cells in resuspension was similar. The rate of loss of non-viable cells from transfusions in which the eventual survival was better than 80 per cent was slow and relatively constant. At 80 per cent survival, or better, the greater part of the loss occurred during the first 300 to 500 minutes from the start of the transfusion, there being, in general, little further loss during the remainder of the first 24 hours.

In transfusions, the survival of which was less than 80 per cent, the rate of loss of non-viable cells was rapid and tended to vary directly with the eventual survival. The rate of loss was greatest during the first 60-minute period, becoming progressively less during the next 24 hours. The

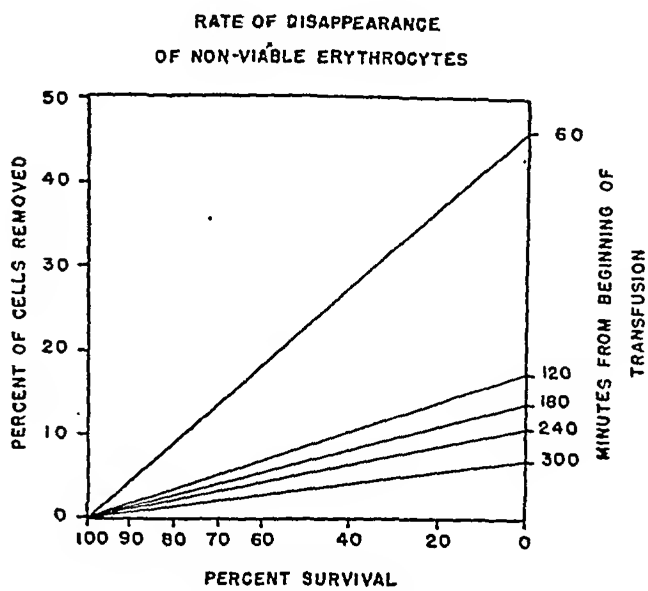

FIG. 3. RATE OF DISAPPEARANCE OF NON-VIABLE ERYTHROCYTES

The average percentages of total transfused tagged red cells removed from circulation during successive 60-minute intervals from beginning of transfusion, in relation to the percentage of survival.

maximum loss was not reached during the first 5 hours, but the rate of loss during the ensuing 19 hours was considerably less than during the first 5-hour period. It should be emphasized that even in the poorest transfusions the 24-hour data still measure disappearance uncomplicated by cell regeneration.

It is therefore apparent that the rate of disappearance of non-viable cells is a function of percentage of survival, which in turn is a function of time of storage.

It is possible, from the time survival curves presented in Figures 1 and 2, to determine, for the entire series of experiments, the average rate of disappearance of cells at given intervals after the beginning of transfusion for any percentage of eventual survival. The family of curves representing these rates at 60-minute intervals up to 5 hours after transfusion are shown, in relation to survival, in Figure 3.

It is evident that the loss of non-viable cells begins very soon after the administration of blood and, in badly deteriorated bloods, may begin during administration. Regardless of the survival, the rate of loss is most rapid during the first 60 minutes' post-transfusion period and decreases considerably during the ensuing four 60-minute periods.

This fact is more clearly evident in Figure 4, in which the average rates of loss of non-viable cells during the first 5 hours after the beginning of transfusion, expressed as per cent of cells lost per minute, are shown for transfusions ranging from 100 to 50 per cent survival.

In the same figure, the total quantity of hemoglobin that would be obtained from *immediate*

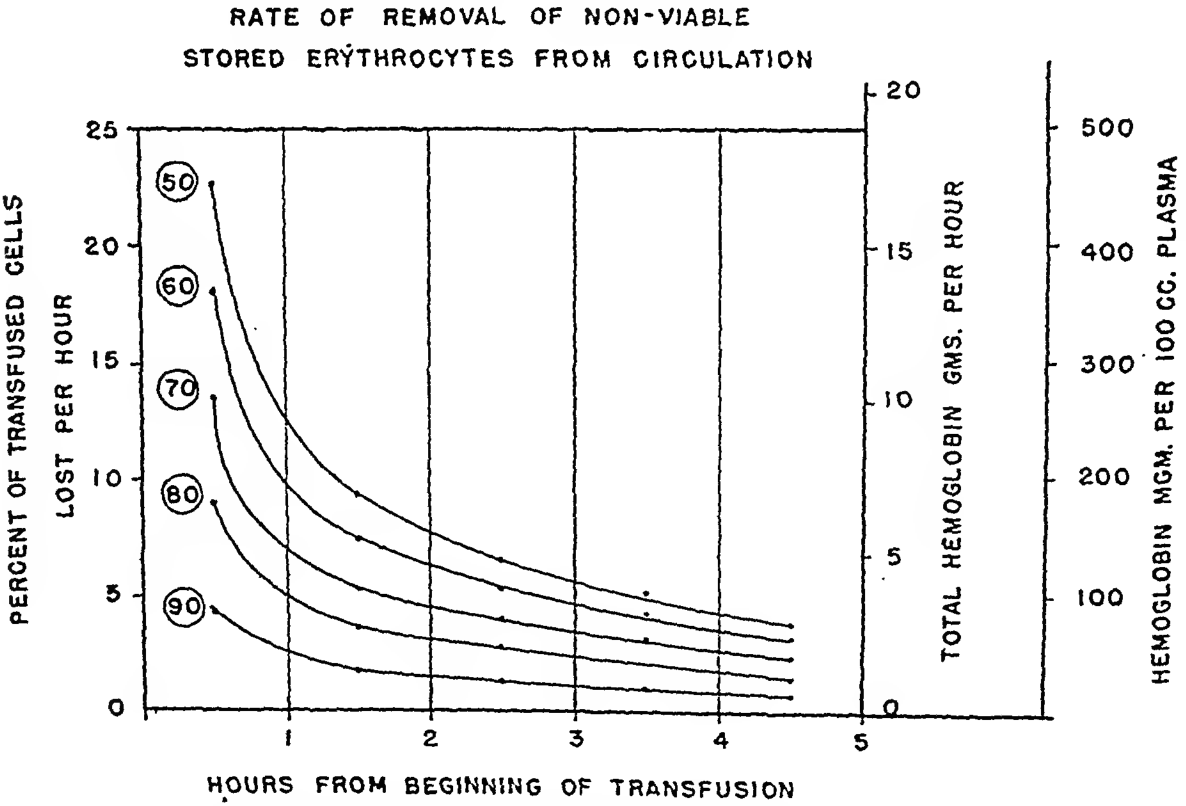

FIG. 4. RATE OF REMOVAL OF NON-VIABLE STORED ERYTHROCYTES FROM CIRCULATION

The average percentages of loss of the total transfused tagged red cells per hour during the first 5 hours from beginning transfusion, in bloods ranging from 50 to 100 per cent survival (indicated by figures in circles). The total amount of hemoglobin, in grams per hour (assuming immediate and complete intravascular hemolysis occurs), liberated from non-viable red cells following transfusion of blood surviving from 50 to 100 per cent, is shown in the first column on the right. The theoretical plasma hemoglobin level, in an individual with a plasma volume of 3,500 ml. resulting from complete retention of the liberated hemoglobin, is shown in the column on the extreme right.

intravascular hemolysis of non-viable cells is shown in relation to the per cent of transfused cells destroyed. These quantities are also plotted as mgm. of hemoglobin liberated per hour per 100 ml. of plasma, in an individual with a plasma volume of 3,500 ml.

Hemoglobinuria was observed only in those of our subjects who received full-sized transfusions of blood the survival of which was less than 50 per cent, and then was a transient affair, the urine being clear within a few hours after transfusion. Even in these subjects the rise in serum total bilirubin did not exceed 2 mgm. per 100 ml. of plasma. It is clear that many dead cells must be removed relatively intact from the blood stream. The role of the spleen in this regard is too well known to elaborate on.

The greatest burden of disposing of cell breakdown products is imposed within the first 2 hours after transfusion has begun. The extent of this load will vary inversely with the size of the transfusion, percentage of non-viable cells, and speed of inflow.

It would appear to be safe practice to set the lower limit of acceptable stored blood at 70 per cent post-transfusion survival. This certainly provides a margin of safety which might not be present were a less good survival accepted.

This concept affords a basis for the assignment of upper dating limit of bloods in the several solutions at present in use in certain blood banks.

Reference has been made (5) to the re-utilization of iron derived from broken down cells in the synthesis of new hemoglobin. It is of interest to know to what extent this blood iron is economized. Twenty-six experiments were completed in which recipient's blood samples were taken at frequent intervals, up to 21 days after transfusion, thus per-

TABLE II

Re-utilization of radio-iron derived from non-viable stored erythrocytes

Solution	Exp. no.	Days stored	Transfused			Per cent of radioactive data		
			Whole blood	Hct.	Cells	Retained	Available	Regen.
			ml.		*ml.*			
McGill II	1	0	157	32	50.3	94	6	4
	2	10	142	29.8	42.3	92	8	6
	3	14	117	31.0	36.0	76	24	11
	4	21	137	31.2	42.6	48	52	30
	5	29	143	31.9	45.6	22	78	52
ACD-1	10	2	84	44.5	37.4	100	0	0
	12	10	77	45.0	34.6	100		
	13	39	82	45.5	36.8	70	30	20
Cells resuspended in saline	6	0	175	40.0	68.0	87	13	5
	7	3	160	42.0	67.2	84	16	6
	8	10	140	46.1	63.7	10	90	32
Cells resuspended in 10 per cent corn syrup	14	1	59	52.0	30.7	100	0	0
	15	5	57	33.5	19.1	60	40	28
	16	12	62	32.7	20.3	43	57	32
	17	21	97	34.2	32.2	17	83	73
DeGowin	18	0	157	25.6	40.4	88	12	5
	19	13	166	24.8	41.2	85	15	3
	20	23	141	27.8	39.2	80	20	14
	21	36	153	19.5	29.8	22	78	71
Parpart's	25	0	120	28.5	34.2	96	4	4
	26	14	118	28.0	33.0	95	5	3
	27	27	126	31.0	39.1	88	12	12
ACD-1	32	15	132	38.7	51.1	85	15	5
	33	29	128	42.6	54.5	51	49	24
	34	41	128	40.1	51.3	37	63	33
		15	132	38.7	51.1	90	10	5
		29	128	42.6	54.5	60	40	18
		41	128	40.1	51.3	42	58	24

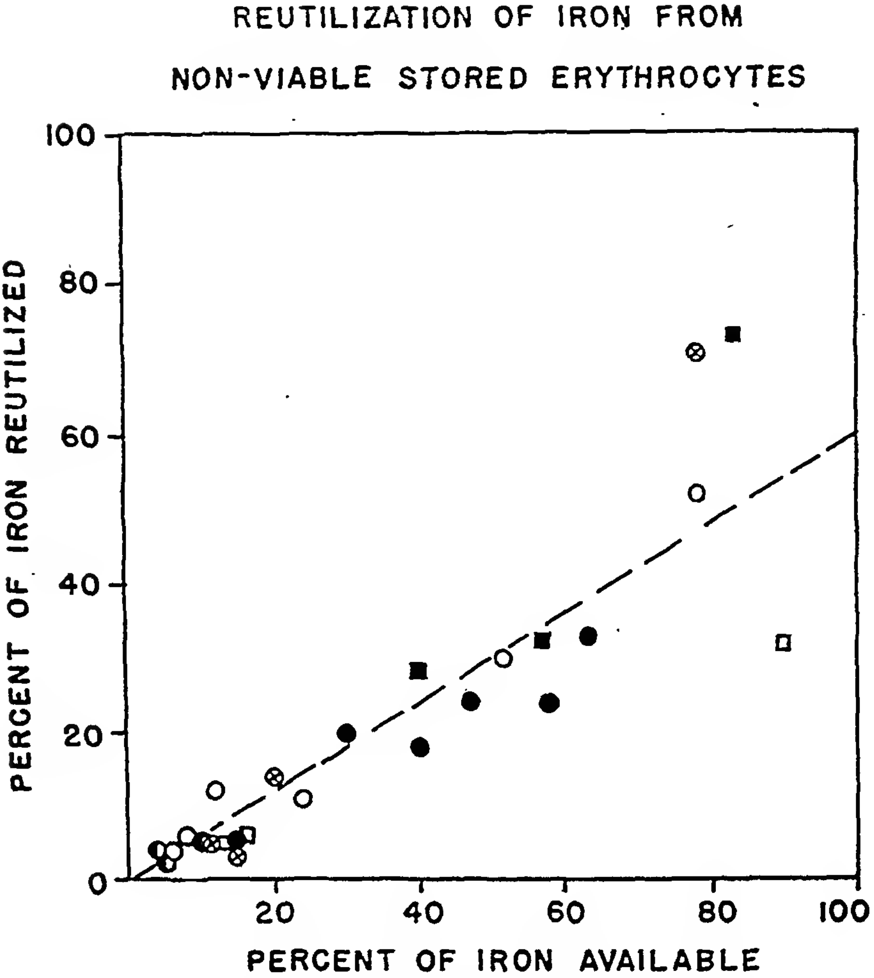

FIG. 5. RE-UTILIZATION OF IRON FROM NON-VIABLE STORED ERYTHROCYTES

The percentage of radio-iron derived from hemoglobin from liberated non-viable tagged transfused erythrocytes found in intact circulating red cells (re-utilized) in relation to the percentage of total transfused cells removed from circulation (available). About 60 per cent of the available iron is economized.

Symbols refer to the following blood preservatives:

● = ACD-1 ⊗ = De Gowin's solution
○ = McGill solution ■ = Cells in 10 per cent corn syrup
◐ = Parpart's solution □ = Cells in 0.85 per cent saline.

mitting measurement of maximum re-utilization. Significant data from these experiments are shown in Table II. In 5 of the series the cells were transfused as whole blood; in 3 as resuspended cells. The quantity of cells given was small, ranging from 19 to 68 ml., averaging 41 ml., the contained hemoglobin, averaging about 15 grams. Individual circulating red cell volumes were from 1,780 ml. to 2,520 ml., averaging 2,214 ml. Survival was from 100 to 17 per cent.

The method of computing the percentage of transfused cells retained, in circulation and regenerated, has been described elsewhere (5). The percentage of iron available for resynthesis is taken as the arithmetic difference between 100 per cent and the percentage retained. These values are listed in Table II. together with the percentage of available iron actually re-utilized.

The percentage of iron re-utilized is plotted against the per cent available, in Figure 5. It is apparent that about ⅔ of the iron does eventually return to the blood stream in the hemoglobin of

new circulating red cells and that this percentage is relatively constant within the range of the dosage given. Forty ml. of cells contain about 50 mgm. of iron. Hence the iron administered as defunct cells ranged from 0 to 40 mgm.

Hahn, *et al* (6) have demonstrated that the absorption of radioactive iron from the normal gastrointestinal tract, as measured by the amounts detectable in circulating red cells, is extremely small. Cruz (7) also found that radio-iron liberated from hemoglobin from destroyed red cells was utilized nearly quantitatively even in the presence of normal iron reserves. It is of considerable interest that iron given intravenously, as hemoglobin in non-viable cells, is utilized approximately 20 times as efficiently as when iron is given orally.

Sufficient data are not available at present to determine the efficiency of utilization when larger amounts of hemoglobin iron are made available. In many experiments, however, in which full transfusions of very much deteriorated cells were given, the recipient's circulating red cell radioactivities have shown utilization of from 10 to 30 per cent of the available iron on the fifth post-transfusion day. Since ½ of the per cent available utilized is usually present on the fourth to seventh post-transfusion day, this would suggest excellent economy of quantities at least 10 times those dealt with in the above experiments.

CONCLUSIONS

(*1*) Non-viable stored human erythrocytes are rapidly removed from the blood stream after transfusion.

(*2*) The rate of removal of non-viable cells varies inversely with the percentage survival of the transfused tagged cells.

(*3*) At or above 80 per cent survival, non-viable cells are completely removed in 24 hours; below this survival level, loss of non-viable cells may continue into the second post-transfusion day.

(*4*) At any survival level, the majority of non-viable cells are removed from the blood stream during the first 2 hours after transfusion.

(*5*) On theoretical and practical grounds 70 per cent retention of all transfused cells may be considered the lowest safe survival level.

(*6*) The bodily economy of iron derived from the hemoglobin of non-viable cells is such that the utilization is about 20 times that of equivalent amounts of iron given orally.

BIBLIOGRAPHY

1. Gibson, J. G., 2nd, Evans, R. D., Aub, J. C., Sack, T., and Peacock, W. C., The post-transfusion surivival of preserved human erythrocytes stored as whole blood or in resuspension, after removal of plasma, by means of two isotopes of radioactive iron. J. Clin. Invest., 1947, 26, 715.
2. Hawkins, W. B., and Whipple, G. H., The life cycle of the red blood cell in the dog. Am. J. Physiol., 1938, 122, 418.
3. Shemin, D., and Rittenberg, D., Studies on the formation of heme and on the average life time of the human red cell. Federation Proceedings, 1946, Vol. 5, Part II, p. 153.
4. Singer, K., and Weisz, L., The life cycle of the erythrocyte after splenectomy, and the problems of splenic hemolysis and target cell formation. Am. J. M. Sc., 1945, 210, 301.
5. Gibson, J. G., 2nd, Aub, J. C., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., and Sack, T., The measurement of post-transfusion survival of preserved stored human erythrocytes by means of two isotopes of radioactive iron. J. Clin. Invest., 1947, 26, 704.
6. Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H., Radioactive iron absorption by gastrointestinal tract. Influence of anemia, anoxia and antecedent feeding distribution in growing dogs. J. Exper. Med., 1943, 78, 169.
7. Cruz, W. O., Hahn, P. F., and Bale, W. F., Hemoglobin radioactive iron liberated by erythrocyte destruction (acetylphenylhydrazine) promptly re-utilized to form new hemoglobin. Am. J. Physiol., 1942, 135, 595.

THE EFFECT OF VARYING TEMPERATURES ON THE POST-TRANSFUSION SURVIVAL OF WHOLE BLOOD DURING DEPOT STORAGE AND AFTER TRANSPORTATION BY LAND AND AIR[1]

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The successful establishment of a military whole blood transfusion service is dependent upon reducing damage to the cells from mechanical agitation incident to land, sea, and air transportation to a minimum. Robertson (1) successfully transported whole blood in Rous-Turner solution by ambulance for short distances in World War I. Blood from civilian donor centers was shipped to front lines during the Spanish Civil War (2). Maycock has reported on the use of blood transported in refrigerated trucks during the Battle of Flanders (3). During the invasion of Europe blood banks were in operation in many theatres, blood being obtained from military personnel, a necessarily limited source of supply.

The establishment of the American Red Cross Blood Donor Service, taking blood for processing of plasma, afforded a potentially adequate supply source, provided blood could withstand shipment across the Atlantic and far out into the Pacific oceans. The distances involved were so great that air transport was the only feasible means of transportation that would get blood to medical personnel before it had seriously deteriorated.

It was obvious that only the best known whole blood preservatives would suffice, and 2 solutions, Alsever's (4) and acid-citrate-dextrose (5), were under consideration by the Armed Forces at the time this study was undertaken. The comparative value of these 2 solutions for depot storage under constant refrigeration has been reported (6), ACD being superior to Alsever's. The survival of these cells in these preservatives after long-range shipment, with and without controlled refrigeration, was not, however, known.

De Gowin (7) in 1941, transported whole blood in a modified Rous-Turner solution in vacuum collecting bottles for distances up to 720 miles by automobile, and up to 3,500 miles by airplane. The bottles were refrigerated with cracked ice which was replaced, if melted, every 12 hours. These bloods were transfused without reactions, although no post-transfusion survival studies were made. Marked destruction of cells as evidenced by hemolysis did not occur. Kendrick, *et al* (8) concluded that "blood collected in Alsever's solution, after adequate pre-chilling (24 hours), could be flown unrefrigerated for 24 hours from the United States to the United Kingdom and would be safe to use for 21 days after collection, that is, when the blood is refrigerated continuously following its arrival in the United Kingdom."

These experiments were designed to define the optimal temperature of refrigeration and the effect of uncontrolled refrigeration both during depot storage and during land and aerial transportation. The effects of extreme ranges of temperature, from $-4°$ C. to $+40°$ C., were studied in bloods in both ACD-1 and Alsever's solutions stored in depots. Since the problem of successful transportation involved the determination of adequate refrigeration range, experiments were conducted in which bloods were transported by air both under controlled and uncontrolled refrigeration.

The method of measuring post-transfusion survival by means of 2 isotopes of radioactive iron has been previously described (9, 10).

DEPOT STORAGE EXPERIMENTS

In all these experiments blood from the same donor was taken in equal amounts into both solutions (except

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts Institute of Technology, in collaboration with the Peter Bent Brigham Hospital.

Experiments 51 through 55), and aliquots of each were transfused after storage for equal periods of time under identical temperature conditions.

Experiments 65 (Alsever) and 66 (ACD)

The aliquots of blood were stored at 4° C. for 4 hours and then removed to a constant temperature apparatus and kept at −4° C. for 24 hours. They were then returned to storage at 4° C. until transfused, the blood in ACD at 8 days and that in Alsever's solution at 9 days after drawing. Each transfusion contained about 50 ml. of cells. The blood did not freeze and there was only slight hemolysis in the supernatant fluid. Survival in both experiments was about 75 per cent. Neither recipient experienced any reaction.

Experiments 57, 59, 61 (Alsever) and 56, 58, 60 (ACD)

The aliquots of this blood were kept at 4° C. throughout the whole storage period and were transfused at 11, 15, and 21 days after drawing, described above in the section on whole blood preservatives. Survival in both solutions was satisfactory (70 per cent or better) up to 21 days.

Experiments 63 (Alsever) and 62 (ACD)

Bloods were stored 46 hours at 4° C., then allowed to stand at room temperature (20 to 25° C.) for 24 hours, and returned to storage at 4° C. until transfused 18 days after drawing. Each transfusion contained about 100 ml. of cells. The recipient of the ACD blood had a mild transient febrile reaction, and rabbit pyrogen test on the supernatant was positive. The other recipient had no reaction and the pyrogen test was negative. Survival of blood in ACD was better than that in Alsever's, but both were less good than was obtained for bloods in both solutions stored at 4° C. for 18 days.

Experiments 67 (Alsever) and 64 (ACD)

Bloods were stored at 4° C. for 4 hours, then transferred to an incubator where they remained at +40° C. for 24 hours, and returned to storage at 4° C. until trans-

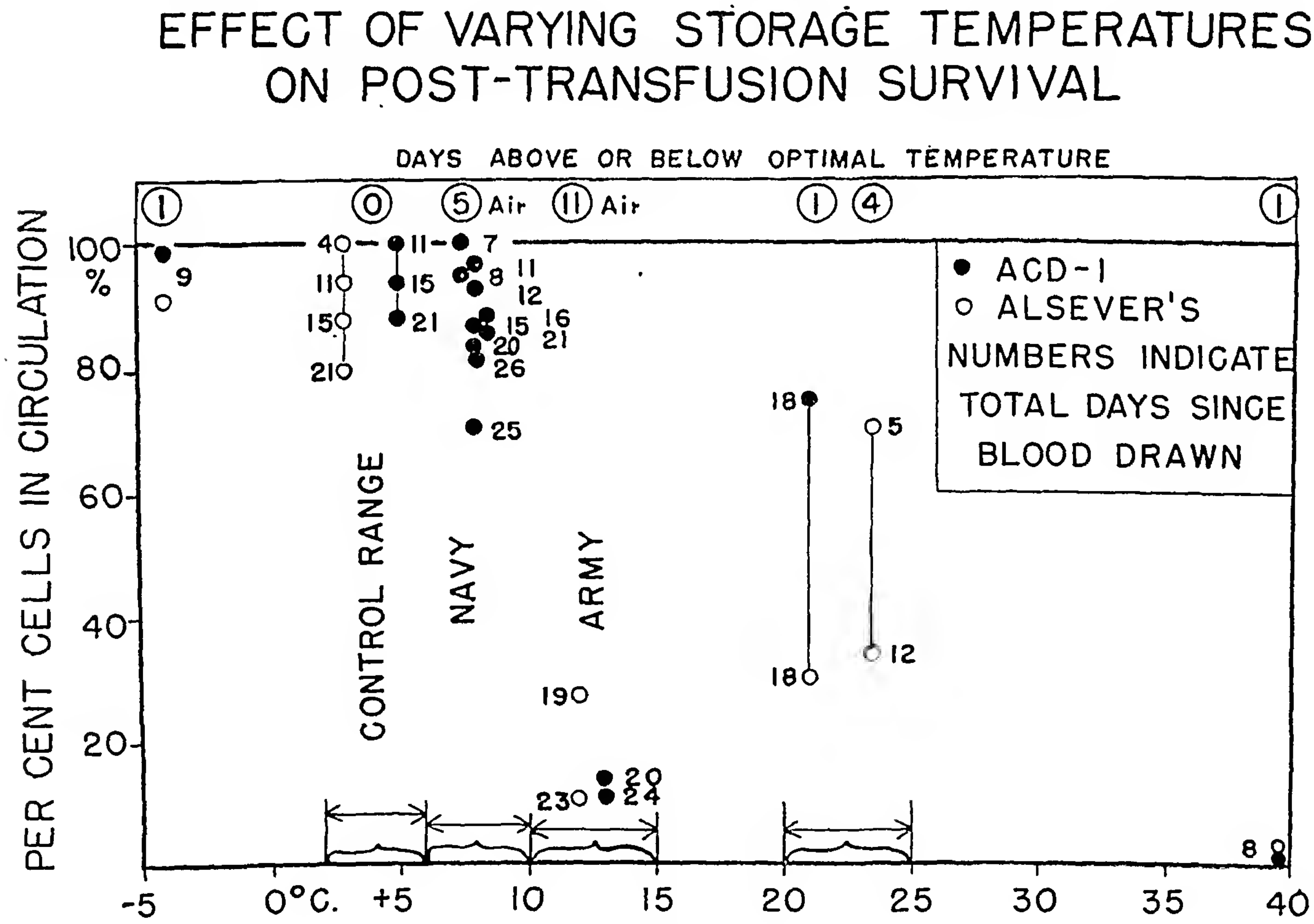

Fig. 1

Figures within circles indicate the days bloods were at, above, or below the optimal (control) range. In all instances the bloods were returned to storage at 4 to 6° C. after exposure to the test temperatures. Aerial transportation, with adequate refrigeration, is not deleterious. Lack of adequate refrigeration is deleterious both during aerial transport and subsequent optimal refrigeration. Exposure to room temperature for even short periods is harmful.

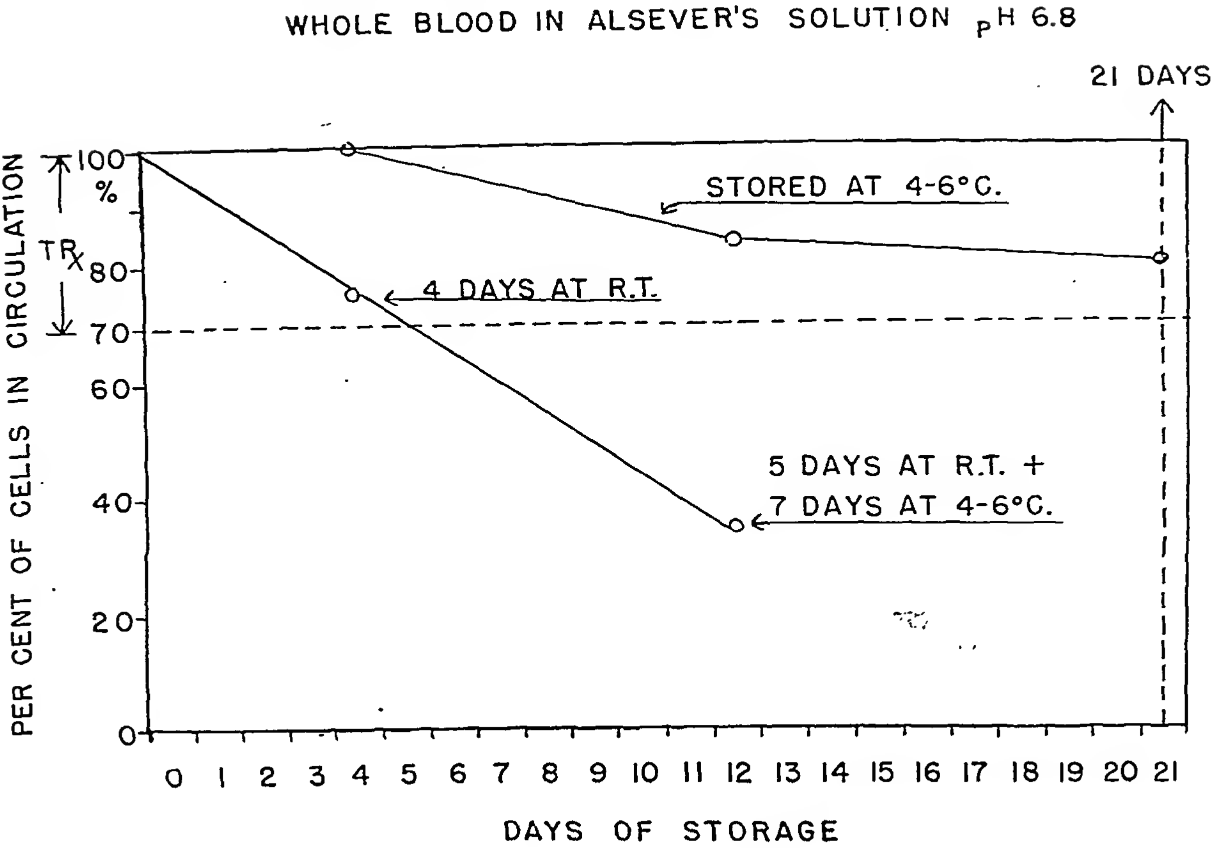

FIG. 2

All aliquots of blood in Alsever's solution were drawn from the same donor. Storage for 4 days at room temperature (20 to 25° C.) initiated deteriorative changes, as evidenced by the superior (25 per cent) survival of the aliquot in depot storage at 4° C. for 4 days, the rate of which was not retarded by subsequent refrigeration at 4° C.

fused 8 days (Alsever's) and 7 days (ACD) after drawing. Each transfusion contained about 80 ml. of cells. The supernatants of both bloods were deeply hemolyzed, but neither recipient had a reaction. Virtually no radioactive cells were found in the blood stream of either recipient 24 hours after transfusion.

The survival data obtained in these depot storage experiments are shown in Figure 1.

Experiments 51 through 55 (Alsever)

These experiments were designed to determine whether deteriorative changes that might occur at room temperature could be arrested by subsequent storage at lower ranges.

Blood from 1 donor was taken into Alsever's solution and divided into 5 equal aliquots. Three of these were transfused after storage at 4° C. for 4, 12, and 21 days after drawing. Another was transfused after storage at room temperature (20 to 25° C.) for 4 days. The other was kept at room temperature for 5 days, then stored at 4° C., and transfused 12 days after drawing. Each transfusion contained about 50 ml. of cells. No reactions occurred.

As shown in Figure 2, survival after 4 days at room temperature was markedly less than after 4 days at 4° C., and even worse after subsequent refrigeration at 4° C. for 7 additional days than was the control stored at depot temperature.

TRANSPORTATION EXPERIMENTS

Two series of experiments were carried out; in one, blood was taken into ACD and transported under controlled refrigeration,[2] while, in the other, bloods in both ACD and Alsever's solution were transported under uncontrolled refrigeration.[3] In both experiments, the bloods were flown about 6,000 miles, in the first overland and in the second overseas.

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[2] This experiment was undertaken at the request of the U. S. Navy, Medical Corps, in cooperation with Capt. Lloyd Newhouser, U.S.N., M.C.

[3] This experiment was undertaken at the request of the U. S. Army, Medical Corps, in cooperation with Capt. John Elliott, A.U.S., Sn.C., Capt. John Reichel, A.U.S., M.C., and Capt. Ellis Vaubel, A.U.S., M.C.

Controlled refrigeration. Experiments 68 through 77

Full bleedings (480 ml.) were taken on the same day from each of 10 group O, Rh positive radioactive donors into standard 600 ml. vacuum bottles containing 120 ml. of ACD and were immediately refrigerated at 4° C. The

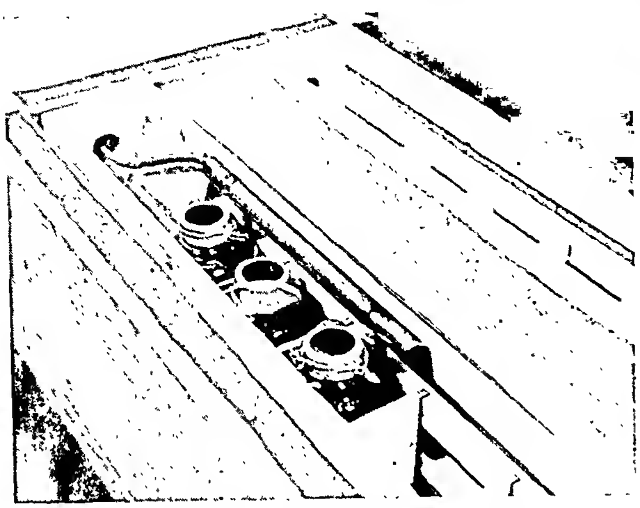

Fig. 3A. Refrigerator Used for Aerial Transport of Whole Blood in ACD-1

The temperature-sensitive element of the gas-filled recording thermometer was fixed in position in close proximity to the bottles of blood. Note also the dioxane indicators fastened to the bottle necks.

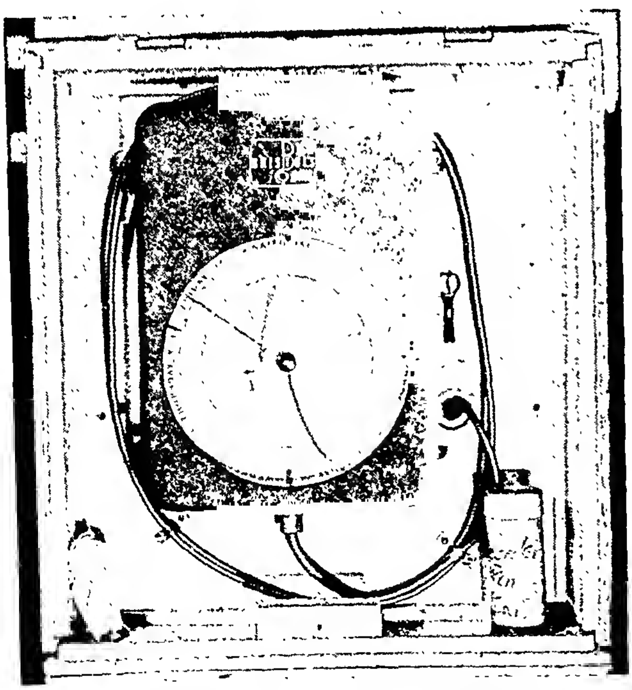

Fig. 3B. Refrigerator Used for Aerial Transport of Whole Blood ACD-1

Bristol recording thermometer, mounted on icebox. A continuous recording was obtained during the 5 days en route.

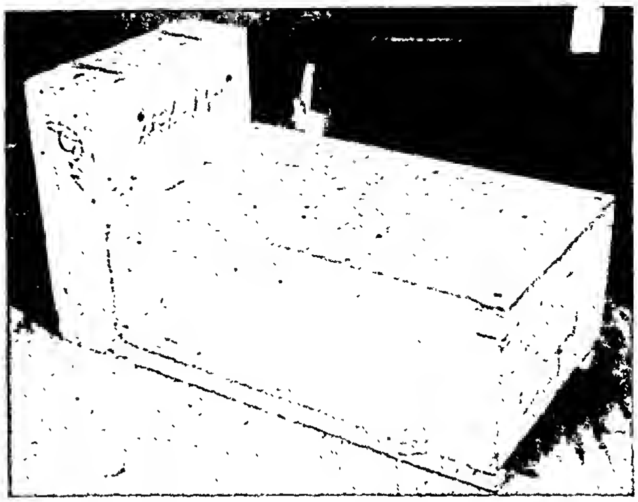

Fig. 3C. Refrigerator Used for Aerial Transport of Whole Blood ACD-1

Refrigerator on return from Oakland, California.

refrigerator (Figure 3) in which these bloods were transported was made for this experiment at the Naval Medical Center, Bethesda, Maryland, and consisted of an insulated wooden chest, with 12 compartments for blood bottles and a single large central compartment for water ice and a tightly fitting insulated cover. A Bristol gas-filled recording thermometer was attached to the refrigerator in such a way that the thermal element lay between the 2 rows of bottles. A thermal indicator was attached to the neck of each bottle. These were "L" shaped glass bulbs containing dioxane, the melting point of which is + 10° C. They were frozen with the dioxane all in 1 arm of the tube and so mounted on the bottle that the frozen arm was uppermost. Exposure to temperatures above 10° C. melts the dioxane which then flows into the lower arm, where it remains even though re-frozen.

This shipment of blood went by National Air Transport Service from the East Boston Airport to the National Airport at Washington; from there to Oakland, California, and was returned by National Air Transport Service from Oakland directly to Boston. The package was placed in a cold room at the Naval Medical Center in Bethesda, Maryland, and also at Oakland while awaiting air transportation. The time elapsed between leaving from and returning to Boston was 122 hours. The package was in flight 22 hours, and in mechanical refrigerators for 34 hours. During the remaining 66 hours, the only refrigerant was the ice in the box itself. The box was re-iced once in Oakland during the 5-day trip. The distance covered by air was about 6,000 statute miles.

The recorded temperatures (Figure 4), when corrected for variations in altitude during flight, did not exceed 15° C. at any time during the trip, and 6 of the dioxane indicators were melted and 4 still frozen, with the dioxane in the upper arm of the tube, on arrival. They were promptly returned to depot storage at 4° C.

The condition of these bloods, as determined by *in*

TABLE I

Condition of human erythrocytes transported in ACD and Alsever's solution under continuous and intermittent refrigeration, as determined by in vitro *tests*

Exp. no.	Days since drawn	Transfused: Whole blood	Transfused: Red cells	Total Hb	Supernatant: Hb*	Supernatant: Cells hemolyzed**	Supernatant: pH	Cells hem. in 0.6 per cent NaCl	Survival by radio-iron
		ml.	*ml.*	*grams*	*mgm. per cent*	*per cent*		*per cent*	*per cent*
Continuous refrigeration 4 to 10° C.—ACD									
68	7	563	220	65.3			7.04	18	94
69	8	542	247	70.2	36	0.15	7.17	10	89
70	11	552	243	65.8	196	0.92	6.97	12	90
71	12	556	233	66.3	19	0.07	6.94	12	87
72	15	538	230	67.0	51	0.24	6.97	15	81
73	16	565	253	72.5	58	0.25	7.20	24	83
74	20	558	234	56.6	74	0.42	7.02	26	81
75	21	568	246	68.8	112	0.52	7.01	18	83
76	25	326	150	38.7	115	0.52	6.95	22	72
77	26	284	125	27.6	159	0.44	7.12	28	78
Intermittent refrigeration 4 to 15° C.—Experiments 78, 80 in Alsever's; 79, 81 in ACD									
78	19	575	161	44.3	79	0.75	6.70	50	27
79	20	366	156	41.3	166	0.85	6.70	63	13
80	23	515	151	40.8	109	1.09	6.65	83	10
81	24	285	122	31.6			6.80	70	9

* Determined by hemochromogen technic.
** Percentage of total cells corresponding to quantity of hemoglobin in supernatant by the equation

$$\text{per cent cells hemolyzed} = \frac{\text{ml. supernatant} \times \text{Hb. in mgm. per cent}}{\text{Total Hb. in mgm.}}.$$

vitro studies carried out just prior to transfusion, is shown in Table I. Very little hemolysis had occurred, amounts present in the supernatant of a centrifuged sample representing hemolysis of less than 1 per cent of the total cells drawn. The pH of the supernatants ranged from 7.2 to 6.94. Fragility tests showed from 10 to 28 per cent hemolysis in 0.6 per cent NaCl.

These 10 bloods were transfused into group A, Rh positive individual recipients, each of whom had previously been bled 500 ml., at intervals of from 7 to 26 days after drawing. All the blood in the bottle was given except in the cases of the 25- and 26-day-old bloods in which about ½ of the contents was given. Two recipients, with allergic histories, experienced anaphylactoid reactions, and 3 exhibited symptoms which could be attributed to rapid breakdown of red cells. Subsequent analysis of data in one of these receiving a high titre blood, indicated that his own cells had been destroyed (10).

As shown in Table I and Figure 1, the post-transfusion survival of these bloods was not less than 80 per cent up to 21 days after drawing, and not less than 70 per cent in the two oldest bloods.

Uncontrolled refrigeration. Experiments 78, 80 (Alsever); 79, 81 (ACD)

Five hundred ml. of whole blood from each of 5 donors were taken into 500 ml. of pre-chilled Alsever's solution in standard 1,000-ml. vacuum bottles, and 480 ml. of whole blood from each of 5 donors into 120 ml. of pre-chilled ACD in standard 600-ml. vacuum bottles. All donors were group O, Rh positive, and were bled the same day. The bloods were refrigerated immediately after drawing. The bottles were prepared for shipment in a manner simulating that in routine use at the time by the Army Medical Corps for overseas aerial transport. They were replaced in the manufacturer's cartons which were then wrapped in heavy brown paper and sealed with gummed tape. Just prior to this, a dioxane (M.P. 10° C.), caprylic acid (M.P. 15° C.) and a water (F.P. −2° C.) thermal indicator was attached to the neck of each bottle. The 2 cartons, one containing blood in Alsever's and the other blood in ACD, were fastened together and placed in a light wooden box attached to the same recording thermometer used in the previously described experiments, the thermal element being enclosed within the cartons.

This shipment was flown from Boston to La Guardia Field, N. Y., and thence by Air Transport Service to Paris, where it arrived 6 days later. The recorded temperature was 15.6° C. and the package was refrigerated at 3.3° C. in Paris, for 31 hours. It was then flown back to La Guardia Field where it was again refrigerated until returned to Boston. It was en route 11 days. No refrigeration was used during air passage.

Immediately on arrival the bottles were refrigerated at 4° C. All of the dioxane, 5 of the caprylic acid, and all of the water indicators were melted. The exact number of hours the package had been in flight, in refrigerators, or unrefrigerated is not known. The graph of the recording thermometer was blurred so that no readings could be obtained.

The conditions of these bloods, as determined by *in vitro* tests carried out just prior to transfusion, is shown in Table I. Very little spontaneous hemolysis occurred. The pH of the supernatants ranged from 6.65 to 6.80, and hemolysis in 0.6 per cent NaCl from 50 to 83 per cent.

Two bloods in half amounts in Alsever's were transfused at 19 and 23 days, and 2 in ACD at 20 and 24 days after drawing. Each transfusion contained about 125 ml. of cells. The remaining bloods were not transfused. One recipient experienced moderately severe symptoms of rapid breakdown of cells but without lasting sequelae. Post-transfusion survival ranged from 9 to 27 per cent, as shown in Figure 1.

DISCUSSION

It is evident from Figure 1 that the optimal survival of cells in both solutions, in depot storage,

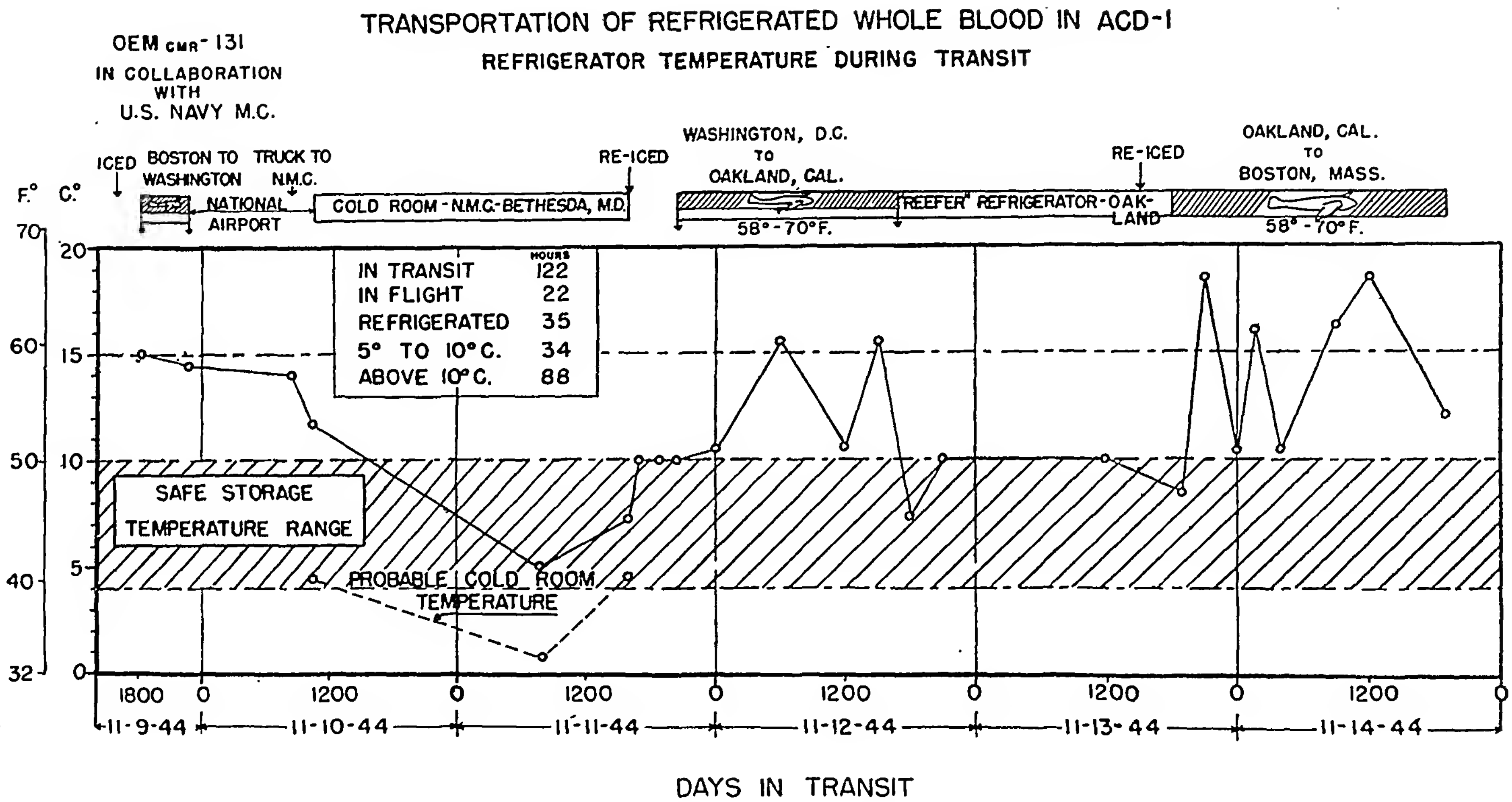

FIG. 4. BLOODS IN BOTH ACD-1 AND ALSEVER'S SOLUTION WERE TRANSPORTED BY AIRPLANE ABOUT 6,000 MILES AS DESCRIBED IN THE TEXT

The cross-hatched bars indicate the time elapsed from leaving and returning to our laboratory; the open bars, the period in depot storage at 4° C. after return. The survival of the bloods transported under controlled refrigeration was equal, at any given observation period, to that of whole blood in ACD-1 in depot storage at 4° C. None of the bloods transported under uncontrolled refrigeration were fit for transfusion, in spite of a period of depot storage at 4° C.

was obtained in the series stored at 4° C. Slight deterioration was evident in the cells stored at − 4° C., much more at room temperature, while complete deterioration occurred at + 40° C. The results obtained in Experiments 51 through 55 indicate that once deterioration is initiated at room temperature, it is not retarded by subsequent storage at lower temperatures, as shown in Figure 2. The data in Figure 2 also indicate a slightly superior ability of cells in ACD to stand up under adverse thermal conditions than in Alsever's solution.

It is apparent that controlled refrigeration at not over 10° C. is an essential for the satisfactory preservation of cells during transportation.[4] No significant deterioration of cells in ACD–1 resulted from the mechanical agitation incident to land and air travel, in the experiment in which temperature was maintained with a fair degree of constancy at about 10° C., since the survival after 5 days of travel and 16 days of subsequent refrigeration was as good as that obtained for blood in the same solution after depot storage for a similar period.

It is also evident that lack of constant refrigeration had extremely injurious effects on red blood cells taken in either ACD or Alsever's solution. The results that would have been obtained had the transportation period been 11 days instead of 5 in the first experiment cannot be known, and unfortunately none of the bloods shipped without controlled refrigeration could be transfused until 19 days after drawing. It was stated above that deterioration is accelerated at temperatures in the region above 15° C. and that this rapid deterioration is not slowed by subsequent cooling. The excellent preservation of the ACD bloods would therefore suggest that little damage was done during transport, since the bloods were in depot storage for from 2 to 21 days after their return, ample time for progressive changes to have continued, had they been initiated during flight.

Evidence was obtained that, in the second experiment, the temperature around the bottles had exceeded 15° C. (melted caprylic acid indicators), and itinerary records obtained make it clear that the package was exposed to temperatures as low as 4° C. at various times. Hence, the bloods were subjected to a fluctuating rather than evenly maintained temperature. It seems probable that marked damage was initiated during transportation and continued during subsequent depot storage.

Because of the extreme degeneration, no comparison between the value of the 2 preservatives during transportation at high temperatures can be made.

In a previous paper (11), we stated that no serious danger to a recipient need result from a full transfusion of stored blood of which at least 70 per cent of the cells remained viable, since the rate of destruction of the non-viable cells was slow, but that hemolysis of cells might occur at a rate and in quantities sufficient to raise the plasma hemoglobin level well above the renal threshold if less than 70 per cent of the transfused cells were

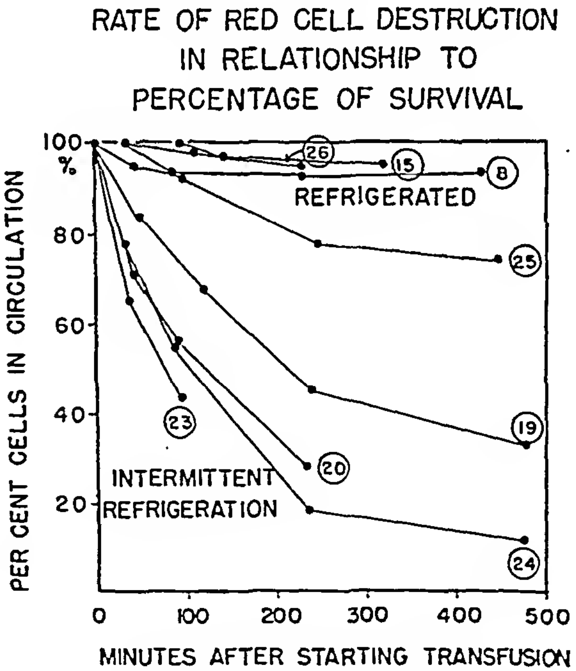

FIG. 5. RATE OF RED CELL DESTRUCTION IN RELATIONSHIP TO PERCENTAGE OF SURVIVAL

The rate of removal of non-viable tagged cells from the blood stream was far less in the refrigerated bloods, the preservation of which was good, than in the intermittently refrigerated bloods, the preservation of which was poor. The figures in the circles refer to the number of days elapsed from drawing to transfusion of blood.

[4] The authors wish to state clearly that no adverse criticism of the performance of the U. S. Army M.C. in flying thousands of bloods in Alsever's solution to E.T.O. is implied. The experiments cited do, however, illustrate the effects of 2 different ranges of temperature control during transportation.

viable. Figure 5 shows the rate of disappearance of cells from the recipient's circulation in both ACD–1 and Alsever's solution, transported under adequate and intermittent (inadequate) refrigeration. It is evident that the greater part of the non-viable cells are eliminated during the first 4 hours after transfusion and that a heavy hemoglobin load may have been placed on those subjects who received the inadequately refrigerated bloods.

The *in vitro* tests were of some value. The relationship of survival as measured by radio-iron and as predicted from changes in osmotic fragility (100 per cent – per cent hemolysis in 0.6 per cent NaCl) was quite good. The supernatant of the refrigerated bloods was slightly alkaline or acid, that of the intermittently refrigerated bloods definitely acid, reflecting a greater production of organic acids in the latter.

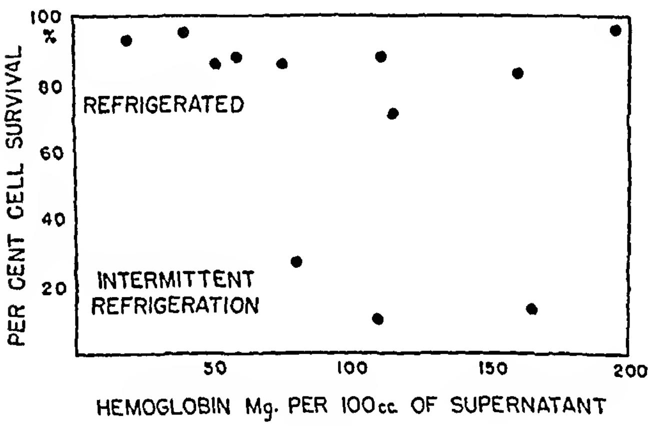

FIG. 6

No correlation between the degree of spontaneous hemolysis, as evidenced by the concentration of hemoglobin in the supernatant plasmas, and the post-transfusion viability of the non-hemolyzed cells was observed in these experiments. The absence of supernatant hemolysis is no criterion of transfusibility. It does not follow that badly hemolyzed bloods can be safely transfused.

A striking fact is the complete lack of correlation between the degree of hemolysis and the measured survival. This is clearly shown in Figure 6. Supernatant hemoglobin ranged from 30 to 196 mgm. per cent in the refrigerated bloods, and from 79 to 166 in the intermittently refrigerated bloods, and yet survival was excellent in the former and extremely poor in the latter series. This does not imply that markedly hemolyzed bloods will have good survival but does demonstrate that absence of spontaneous hemolysis is no indication that stored blood in either ACD–1 Alsever's solution is fit for transfusion.

CONCLUSIONS

(*1*) Refrigeration is an absolute essential f preservation of human erythrocytes drawn a whole blood in either ACD–1 or Alsever's solu tion.

(*2*) The optimal range for depot storage lies between 4° C. and 10° C.

(*3*) Exposure to temperatures below – 4° C. or above 10° C., even for a 24-hour period, increases the rate of *in vitro* deterioration; and this rate is markedly accelerated at temperatures above 15° C.

(*4*) The rate of deterioration resulting from storage at temperatures outside of the optimal range is not retarded by subsequent storage at 4 to 6° C.

(*5*) Whole blood in ACD–1 was transported under refrigeration within the optimal temperature range for 6,000 miles by air; and post-transfusion survival was as good up to 26 days as whole blood in ACD–1 maintained in depot storage at 4° C. for a similar period.

(*6*) Transportation of blood in either ACD–1 or Alsever's under variable temperatures 4 to 15° C. may lead to rapid deterioration of cells in spite of subsequent storage at the point of arrival under optimal refrigeration.

(*7*) The absence of spontaneous hemolysis in bloods transported under controlled or intermittent refrigeration should not be regarded as evidence that such bloods can be safely transfused.

BIBLIOGRAPHY

1. Robertson, O. H., Transfusion with preserved red blood cells. Brit. M. J., 1918, 1, 691.
2. Jorda, F. D., Barcelona transfusion service. Lancet, 1939, 1, 773.
3. Maycock, W. D., Blood transfusion in the B. E. T. Brit. M. J., 1940, 2, 467.
4. Gwynn, C. A., and Alsever, J. B., The collection and preservation of placental blood for transfusion purposes. Am. J. M. Sc., 1939, 198, 634.
5. Loutit, J. F., Mollison, P. L., and Young, I. M., Citric acid-sodium citrate-glucose mixtures for blood storage. Report to Medical Research Council from Southwest London Blood Supply Depot. Quar. J. Exper. Physiol., 1943, 32, 183.
6. Gibson, J. G., 2nd, Evans, R. D., Aub, J. C., Sack, T., and Peacock, W. C., The post-transfusion survival of preserved human erythrocytes stored as whole blood or in resuspension, after removal of plasma,

by means of two isotopes of radioactive iron. J. Clin. Invest., 1947, 26, 715.

7. De Gowin, E. L., and Hardin, R. C., A plan for collection, transportation and administration of whole blood and plasma in warfare. War Med., 1941, 1, 326.
8. Kendrick, D. B., Jr., Elliott, J., Reichel, J., Jr., and Vaubel, E. K., Supply of preserved blood to European theater of operations. Preliminary report. Bull. U. S. Army M. Dept. (No. 84), 1945, 3, 66.
9. Peacock, W. C., Evans, R. D., Irvine, J. W., Jr., Good, W. M., Kip, A. F., Weiss, S., and Gibson, J. G., 2nd, The use of two radioactive isotopes of iron in tracer studies of erythrocytes. J. Clin. Invest., 1946, 25, 605.
10. Gibson, J. G., 2nd, Aub, J. C., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., and Sack, T., The measurement of post-transfusion survival of preserved stored human erythrocytes by means of two isotopes of radioactive iron. J. Clin. Invest., 26, 704.
11. Gibson, J. G., 2nd, Peacock, W. C., Evans, R. D., Sack, T., and Aub, J. C., The rate of post-transfusion loss of non-viable stored human erythrocytes and the re-utilization of hemoglobin derived radioactive iron. J. Clin. Invest., 1947, 26, 739.

ANTITULARENSE SERUM: CORRELATION BETWEEN PROTECTIVE CAPACITY FOR WHITE RATS AND PRECIPITABLE ANTIBODY CONTENT

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Heretofore all attempts to demonstrate protective antibody in antitularense serums have failed whenever animals were challenged with a strain of high virulence. Previous test animals have been the mouse, guinea pig, hamster, and rabbit. Sources of immune or hyperimmune serums were goats, horses, sheep, rabbits, and man. Our considerable unpublished experience with these animals and serums is in good agreement with the reports of Francis and Felton (1) and Bell and Kahn (2). If injected with serum before challenge these animals will usually exhibit significant prolongations of the survival time beyond that of control animals, but no actual survivals against as little as 1 to 10 M.L.D. of a virulent challenge strain.

The inability to demonstrate serum protection in these highly susceptible animals has contributed to a widespread but unfounded belief that immune and hyperimmune serums are ineffective therapeutic agents in human tularemia. Although reports by Foshay (3, 4) on the results of serum therapy in individual patients, as well as analyses of accumulated data derived from the independent observations of more than 600 clinical observers, have continued to demonstrate significant reductions in mortality and highly significant reductions in morbidity in comparison with comparable data from untreated patients, universal acceptance of the value of serum therapy has been impeded by a lack of suitable means to demonstrate protective antibody and to establish quantitative criteria for potency. The work here reported was undertaken to supply these deficiencies.

The inability of the highly susceptible mouse, guinea pig, hamster, and rabbit to react with any useful degree of resistance before death occurs following infection with a single virulent unit of *Bacterium tularense* makes these animals unsuitable test hosts for serum protection experiments. The reasons for this inability, obviously inoperative for many other infectious agents under similar conditions of testing, are unknown at present. The reactions of these animals to invasion are quite unlike that of man, for whom there is ample evidence that the great majority rapidly develop a high degree of resistance, resulting in a disease that is characterized clinically by a low mortality and, pathologically, by tissue reactions that soon exhibit subacuteness and chronicity.

During the course of the initial investigations on this disease, McCoy (5) noted that rats, both gray and white, were highly resistant to cutaneous infection and considerably resistant to subcutaneous inoculation. Infection was proved by demonstration that the spleens of rapidly recovered rats harbored the virulent organisms. Since the reaction to invasion by the rat more closely approximates that of man than do those of other laboratory animals, the rat was chosen as the test animal for serum protection studies.

MATERIALS AND METHODS

Rats. All rats employed were of the Wistar strain. Special care was taken to secure groups of animals of similar weight in order to insure uniformity of results. The permissible variation in weight was from 70 to 115 grams, and the great majority varied between 85 and 110 grams.

Challenge strain. The strain of *Bacterium tularense* used was the highly virulent strain SCHU, which had been studied extensively and had been maintained frequently in animal passage since its isolation in 1941. Its subcutaneous LD_{50} titers for mice, guinea pigs, and rabbits varied from 9.1 to 9.7/0.5 ml. The LD_{50} titers were determined by the method of Reed and Muench (6). They are expressed as the logarithms of the dilutions, disregarding the minus sign.

Challenge suspensions. Challenge suspensions were prepared from the second of 2 consecutive 16-hour subcultures on glucose glycerol cystine human blood agar. The growth was placed in physiological salt solution and suspended evenly by means of a fine bore pipette. Each

suspension was adjusted to a turbidity of 40 microamperes (M.A. 40) in 18-mm. tubes by means of a reflecting photoelectric comparator. These 10° suspensions were used to make serial decimal dilutions in physiological salt solution.

Challenge dose. The challenge dose for all rats was 1 ml. of M.A. 40×10^{-2} dilution, administered subcutaneously. Many plate counts made by the method of Downs, Coriell, Chapman, and Klauber, as well as numerous virulence titrations in mice, showed that the M.A. 40 suspension contained from 2.5 to 3 billions of viable organisms per ml. Hence, the challenge doses contained at least 25 millions of viable, virulent organisms.

Serums. The serums used had the following histories.

A. Hyperimmune goat serum, pooled from bleedings of 4 bucks that had been inoculated intravenously 3 times a week for 5 months with saline suspensions of living strain SCHU. The initial agglutinin titer was 1:20,480; at the time of rat testing it was 1:5,120. The unconcentrated serum, preserved with merthiolate to 1:30,000, had been stored in 60-ml. quantities at 5° C. This serum was 15 months old at the time of the first protection test.

B. Hyperimmune goat serum, pooled from bleedings of 3 bucks that had been inoculated subcutaneously 3 times a week for 1 month with saline suspensions of living strain SCHU. Each of these animals had been inoculated intravenously during the previous year, for preparation of serum A. The initial agglutinin titer was 1:10,240+; at the time of rat testing it was 1:1,280. Unconcentrated serum was preserved with merthiolate to 1:30,000 and held in 60-ml. quantities at 5° C. This serum was 4 months old at the time of the first test.

C. Hyperimmune horse serum No. 50737, obtained from Sharp & Dohme; pooled from 8 bleedings of 1 horse. This animal had been inoculated intravenously twice a week with formalin-killed suspensions of strain SCHU for 11½ months during 1942. During 1943 it was inoculated intravenously 3 times a week for 2 months with suspensions of living strain SCHU and, similarly, with living suspensions for 4 months during the next year. Thereafter, in order to avoid infection of unprotected personnel, formalin-killed suspensions of this strain were injected intravenously as maintenance doses during the first half of the bleeding period. During the second half of this period maintenance inoculations consisted of intravenous injections of phenol-killed whole cultures of strain SCHU in a semisynthetic soybean hydrolyzate liquid medium. Both unwashed killed suspensions contained about 2.5 billions of bacteria per ml., and included the minute minimal reproductive units of the organism. The weekly dosage was 2 ml. At the time this unconcentrated serum was tested its pooled bleeding aliquots varied in age from 14 to 6 months, and the agglutinin titer was 1:1,280.

D. Aged immune goat serum No. 79409, prepared in 1940–1941 by Sharp & Dohme by intravenous inoculations for 8 months of unwashed heat-killed suspensions of strains of maximal virulence. Pooled serum from 6 goats was preserved with phenol and refrigerated in liquid bulk. At the time of testing it was more than 5 years old, and its agglutinin titer was 1:1,280.

E. Normal horse serum No. 51975, without preservative, obtained from Sharp & Dohme.

F. Normal goat serum prepared from 2 bleedings of an uninoculated kid born of uninoculated parents. One lot contained merthiolate to 1:30,000; the other contained no preservative.

Quantitation of precipitable antibody. The precipitable antibody content of serums was determined by the neutralization method of Culbertson (7). The antigen was a nonimmunogenic polysaccharide prepared from acetone extracted cells of strain SCHU by the phenol extraction method of Palmer and Gerlough (8). Antibody content was usually measured with antigen in steps of 10 micrograms. Serum of low antibody content and dilutions of serums of high antibody content were quantitated with sharp end points with antigen increments in steps of 1 microgram. Antibody content is expressed as mgm. of antigen necessary to exhaust antibody from 1 ml. of serum without resultant antigen excess.

Serum protection tests. Serum protection tests were performed in the following manner. Rats were inoculated in groups of 30 to 32, half of each group receiving one of the potent serums and the other half receiving either the corresponding normal serum or the aged immune goat serum. Each rat was injected intraperitoneally with 2 ml. of undiluted serum and immediately thereafter with the 1-ml. challenge dose, subcutaneously, into the abdominal wall near the groin. The 2-ml. serum dosage was selected with reference to the agglutinin titer curves of pooled serums from normal rats after intraperitoneal injections of an immune goat serum with a titer of 1:5,120. Rats that received 2 ml. of serum had titers of 1:1,280 at 24 hours, and 1:160 at 48 hours. Rats that received 1 ml. of serum had titers of 1:160 at 24 hours, and zero in 1:10 dilution at 48 hours. Deaths were recorded daily for 21 days before release. The infecting strain was recovered from a rat dying on the second or third day from each group, and was used to infect each succeeding group.

Determination of persistence of infection in the spleens of recovered rats. After release all recovered animals were held for sacrifice at weekly intervals from 3 weeks to 3 months after challenge and were tested for residual splenic infection by cultures and by intraperitoneal inoculations into mice of suspensions of ground spleens.

EXPERIMENTAL RESULTS

Preliminary virulence titrations in normal rats. Before undertaking protection experiments the optimal subcutaneous challenge dose was determined by virulence titrations in normal rats weighing 70 to 135 grams. Preliminary subcutaneous titrations in adult rats of 200 to 220 grams showed great irregularity of mortality in respect to challenge doses. Mortality was frequently 100 per

cent from M.A. 40×10^{-1}, 10^{-3}, 10^{-4}, and 10^{-9} dilutions, and only 20 to 60 per cent from the intervening dilutions. An increase in the number of rats used per dilution reduced but did not dispel this irregularity. Although rats are highly susceptible to intraperitoneal challenge, yielding LD_{50} titers/1 ml. equivalent to those obtainable in mice injected either S.C. or I.P./0.5 ml., there is great variation in natural resistance among individual rats that are challenged subcutaneously, and this variable resistance apparently increases with age. Subcutaneous titrations in weight groups of 75 to 95 grams and of 95 to 115 grams revealed less irregularity due to variable individual resistance and gave separate LD_{50} titers of 6.4.

The cumulative mortalities and LD_{50} titers from 6 titrations in rats weighing from 70 to 135 grams are shown in Table I. The LD_{50} titers for the separate titrations were 7.6, 8.4, 5.6, 5.2, 8.0, and 6.0, and the cumulative LD_{50} titer was 6.4. This titer became stabilized between 6.3 and 6.5 only after 90 rats were used. The cumulative intraperitoneal mortalities and LD_{50} titers for rats weighing 70 to 210 grams, with separate titers of 9.3, 8.6, 9.5, 9.7, and 8.4, were obtained from simultaneous titrations with the same dilutions used for the subcutaneous titrations. The cumulative intraperitoneal titer of 9.3 indicates that the rat is about 1,000-fold more resistant to subcutaneous challenge than to intraperitoneal challenge. The relatively minor differences among the intraperitoneal LD_{50} titers indicate that the greater variability observed among subcutaneous LD_{50} titers was caused by differences in individual resistance among rats, and was not owing to lack of uniformity of the separate M.A. 40 suspensions or to errors in decimal dilutions prepared from them. The comparison between subcutaneous and intraperitoneal virulence titrations in mice, many performed simultaneously with the same dilutions, shows that the mouse does not exhibit differences in resistance dependent upon the route of challenge and that the total lack of resistance to experimental infection was shared by all mice.

Since 2 consecutive subcutaneous virulence titrations in the variably resistant rat, 30 and 39 animals being used, with simultaneous intraperitoneal titrations with 9 rats each, gave subcutaneous LD_{50} titers of 8.0 and 5.2, and intraperitoneal titers of 8.4 and 9.7, respectively, it became apparent that virulence titrations, if performed simultaneously with serum protection tests, and with fewer than 90 rats per titration, would not permit calculation of the actual challenge doses received by the protected rats. Calculations made from the separate subcutaneous titrations showed that the challenge dose used in protection experiments might have varied from 1,600 to 250,000 LD_{50} doses. Since it was not feasible to use 90 animals for each titration and protection series, and since the M.A. 40×10^{-2} dilution killed regularly at least 96 per cent of rats of the greater weight range, and at the same time permitted protection of more than 50 per cent of animals in preliminary protection tests, the 10^{-2} dilution was adopted for challenge of all rats of the narrower weight range in the protection experiments. For similar reasons it seemed advisable to express the challenge dose as an average in terms of the cumulative subcutaneous LD_{50} titer. On this basis each rat in the following protection experiments received 25,000 LD_{50} doses.

TABLE I

Virulence titrations with strain SCHU in 70 to 135 grams white rats and in 18 to 25 grams white mice

Comparative mortalities and cumulative LD_{50} titers after subcutaneous and intraperitoneal inoculation. Dosage was 1 ml. for rats, 0.5 ml. for mice.

Animal	Route	Decimal dilutions of strain SCHU from M.A. 40 suspension										Cumulative LD_{50} titers
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
Rat	S.C.	9/9*	30/31	17/20	21/26	15/26	13/24	12/24	10/24	4/21	1/18	6.4
	I.P.							8/8	15/16	11/17	2/14	9.3
Mouse	S.C.							14/14	15/15	10/15	1/15	9.3
	I.P.							25/25	34/34	26/35	9/35	9.4

* 9/9 = 9 of 9 animals injected died of tularemia.

Serum protection of rats in relation to precipitable antibody content. The results of serum protection experiments are given in Table II. Instead of comparing each potent serum with its respective control serum all serums are tabulated in descending order with respect to precipitable antibody content. Each serum was tested on 2 separate occasions, and the number of animals and the mortality ratios are shown for each test in order to indicate the residual effect of variable resistance among rats within a weight range of 85 to 110 grams. These differences are negligible for the most potent serum and for both normal serums. The results showed good correlation between survival percentages and mgm. equivalents of antibody content but little correlation between antibody content and agglutinin titers. The low agglutinin titer and the low precipitable antibody content of the normal horse serum were observed independently and were verified repeatedly, but we have no explanation for their presence.

TABLE II

Correlation between precipitable antibody content of antitularense serums and protective capacity for white rats

No. of rats	Group	Serums	Maximal agglt. titers	Mortality	Survival	Antibody content
					per cent	*mgm. per ml.*
16 16	A	Hyperimmune goat by I.V. inoc.	1:5120	2/16 2/16	87.5	1.10
16 15	B	Hyperimmune goat by S.C. inoc.	1:1280	6/16 2/15	74.2	0.7
16 16	C	Hyperimmune horse by I.V. inoc. no. 50737	1:1280	7/16 3/16	68.7	0.5
15 16	D	Aged immune goat by I.V. inoc. no. 79409	1:1280	9/15 6/16	51.6	0.085
15 15	E	Normal horse no. 51975	1:10	15/15 13/15	6.6	0.001
15 15	F	Normal goat	0	14/15 15/15	3.3	0

Isolation of the infecting strain from recovered rats. Mouse inoculation proved to be more reliable than cultures for the recovery of organisms from suspensions of ground spleens. Infected spleens were demonstrated regularly at 3 weeks after challenge, and with a frequency of 75 per cent at 4 weeks. Thereafter, weekly tests yielded the organisms only twice, from single rats, at the eighth and thirteenth weeks.

DISCUSSION

The decisive results of the serum-protection experiments demonstrated conclusively that protective antibody was present in antitularense serums. The observed differences in mortality between rats that received immune serums and those that received normal serums are significant by inspection. The degrees of significance of the mortality rates from groups A, B, C and D, in comparison with the rate obtained after pretreatment with normal horse serum, are extremely high, all values of p being so small that they are unimaginable figures. Since the virulence titrations in rats having a slightly higher maximal weight limit showed that stability was not reliably secured with fewer than 90 rats, we attach less significance to the observed differences in mortality among groups A, B, C and D. Inspection of the paired mortality ratios for groups B, C and D reveals that variable individual resistance among rats in the narrower weight range was still operative. Hence, it is unlikely that significant differences in potency between these serums could be determined without using a much larger number of rats for each serum. Analysis of the rates for groups A, B and C, in comparison with the rate from group D, showed that no difference was significant, though that between groups D and A barely escaped statistical significance.

The protection test employed is satisfactory to determine the presence or absence of protective antibody. In the above tests it actually gave good agreement between rat survival and antibody content for all serums, and it is perhaps possible that repetition might continue to reveal proportional differences in protective antibody in serums of graded potency in agreement with their respective precipitable antibody contents, but, unless larger numbers of animals were used for each serum, conclusions about the relative potencies are not really justified. It is apparent from the mortality of rats in group D that a very small amount of antibody is sufficient to tip the balance heavily in favor of survival, even against a large challenge dose of a strain of maximal virulence.

The variable resistance of rats to subcutaneous challenge would have necessitated the use of so many animals per serum dilution that determinations of the ED_{50} doses of serums were impractical. The possibility of securing more significant degrees of relative serum protection, with the same or a smaller number of animals, by means of a suitable intraperitoneal challenge dose has not been explored.

Although the protection test described provides a method for the demonstration of protective antibody in antitularense serums it does not in its present form furnish an accurate basis for the establishment of quantitative criteria for potency. Further study of the precipitable antibody content in relation to protective capacity may eventually provide a serologic method to quantitate the potency of serums, thus obviating the use of test animals. Until a satisfactory method is devised temporary safeguards might be adopted. For example, a provisional standard might require a minimum of 1 mgm. equivalent of antibody per ml. of serum or perhaps a survival rate of 75 per cent among rats selected and challenged in accordance with the above specifications.

SUMMARY

Protective antibody was demonstrated in antitularense serums against subcutaneous challenge with an average of 25,000 LD_{50} doses of a strain of *Bacterium tularense* of maximal virulence, using white rats of 85 to 110 grams as the test animals. Good correlation was observed between protective antibody and precipitable antibody content, but not between antibody content and agglutinin titers.

ACKNOWLEDGMENT

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BIBLIOGRAPHY

1. Francis, E., and Felton, L. D., Antitularemic serum. Pub. Health Rep., 1942, 57, 44.
2. Bell, J. F., and Kahn, O. B., Efficacy of some drugs and biologic preparations as therapeutic agents for tularemia. Arch. Int. Med., 1945, 75, 155.
3. Foshay, Lee, Tularemia: A summary of certain aspects of the disease including methods for early diagnosis and the results of serum treatment in 600 patients. Medicine, 1940, 19, 1.
4. Foshay, Lee, A comparative study of the treatment of tularemia with immune serum, hyperimmune serum, and streptomycin. Am. J. Med., 1946, 1, 180.
5. McCoy, G. W., A plague-like disease of rodents. Pub. Health Bull. No. 43. Washington, April, 1911, pp. 53–71.
6. Reed, L. J., and Muench, H., A simple method of estimating fifty per cent endpoints. Am. J. Hyg., 1938, 27, 493.
7. Culbertson, J. T., A quantitative study of the precipitin reaction with special reference to crystalline egg albumin and its antibody. J. Immunol, 1932, 23, 439.
8. Palmer, J. W., and Gerlough, T. D., A simple method for preparing antigenic substances from the typhoid bacillus. Science, 1940, 92, 155.
9. Downs, C. M., Coriell, L. L., Chapman, S. S., and Klauber, A., The cultivation of *Bacterium tularense* in embryonated eggs. J. Bact., 1947, 53, 89.

THE ROLE OF PEPSIN, PEPTIC INHIBITORY SUBSTANCES, AND HYDROCHLORIC ACID IN NORMAL SUBJECTS AND IN THE PRODUCTION OF PEPTIC ULCERS[1]

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INTRODUCTION

There is still disagreement as to the respective roles of the pepsin and hydrochloric acid of gastric juice in the production of duodenal ulcer.

In patients with ulcer, free hydrochloric acid has been generally found to be present in increased quantities (1, 2). The work of Dragstedt (3) and of Mann and Bollman (4) have tended to emphasize the importance of hydrochloric acid.

The occurrence of ulcers following the surgical shunting of alkaline secretions (bile and pancreatic juice) away from those portions of intestines where gastric juice was made to enter, served in part as a basis for their deductions. Dragstedt, in addition, pointed out that the rate of digestion of frog's leg in an acid pepsin solution was dependent on acid concentration and independent of pepsin concentration (5). The production of ulcers by feeding hydrochloric acid to experimental animals also served as a basis for their conclusions. Histamine is a potent secretagogue of hydrochloric acid (6), but not of pepsin (7). Ulcers have been produced in practically all species of animals by injection of histamine in beeswax (8). Cases have even been reported in humans following injections of histamine (9). Frequent success over a long period with therapy employing antacids has been another strong argument in support of this premise.

Vanzant, Osterberg, Alvarez, and Rivers (10) have shown that the secretion of pepsin is enhanced in ulcer patients as compared with normal individuals and that a direct correlation existed between the amount of pepsin in gastric juice and the severity of symptoms. Mullins and Flood (11) and others (12) have confirmed these observations. Schiffrin (13) demonstrated that ulcers could be produced by perfusing isolated loops of jejunum or ileum of cats with pepsin and hydrochloric acid solution. Such ulcers were not produced by perfusion with hydrochloric acid alone. Driver's (14) investigations with dogs have yielded comparable results. Matzner (15) was able to produce ulcers in rats by feeding pepsin-hydrochloric acid mixtures.

In any attempt to resolve the controversy over the comparative importance of acid and pepsin, it is necessary to apply more refined quantitative methods of study and to devise mutually exclusive experimental conditions wherein both factors do not vary in the same direction simultaneously.

Accurate determinations of gastric hydrochloric acid are easily made, but most methods for determining pepsin have been either inadequate or cumbersome (16). The reason for inadequacy lies partially in the method and partially in the failure to take cognizance of the kinetics of the enzyme (pepsin) reaction.

THE KINETICS OF THE ENZYME REACTION

Pepsin acts on proteins, splitting them into proteoses, peptones, and polypeptides, but not to the amino acid state (17). In the case of egg albumen, the molecule is either attacked and broken down to a molecular weight of approximately 1,000, or it is not attacked at all (18). Pepsin has a predilection for certain peptide linkages, especially those involving tyrosine and phenylalanine (19). Unlike linkages are split at various rates (19). Pepsin does not act like some simple inorganic catalysts whose mere presence in traces is sufficient to bring about change. This enzyme reaction has been found to obey the Law of Mass Action, so that the rate of reaction and therefore the amount of substance converted are directly proportional both to the amount of active enzyme and to the concentration of active substrate present. However, the rate of conversion is not uniform for more than a brief period. The reaction rate rap-

[1] Done on a grant from the Otho S. A. Sprague Memorial Institute.

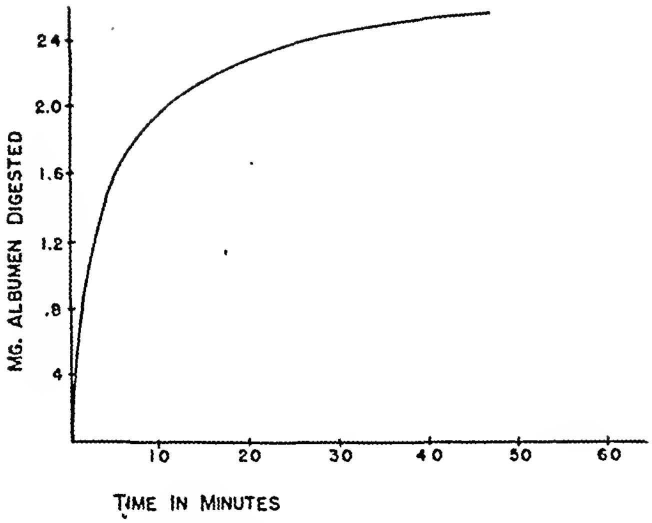

FIG. 1. THE AMOUNT OF PROTEIN DIGESTED IS PLOTTED AGAINST TIME IN MINUTES. THE RATE OF DIGESTION STEADILY DECREASES, REACHING A PLATEAU IN 30 MINUTES

idly diminishes and approaches zero (Figure 1). Peptones,[2] the product of pepsin digestion, combine with the active enzyme to form an inactive pepsin complex. Therefore the quantity of active enzyme present is constantly diminishing as digestion progresses. After the reaction rate has leveled off, the addition of sizeable increment of enzyme fails to produce further digestion, since the small quantity of pepsin initially present has produced large amounts of peptone inhibitor. It is the concentration of these inhibitory substances which determines the amount of pepsin which is active.

This pepsin-peptone complex has also been found by Northrop (20) to obey the Law of Mass Action, so that the following equation applies:

$$\text{Pepsin} + \text{Peptone} \leftrightarrows \text{Pepsin-Peptone}$$

and

$$\frac{\text{Conc. pepsin} \times \text{Conc. peptone}}{\text{Conc. pepsin-peptone}} = K.$$

The equation illustrates the reversibility of the reaction. Thus, if one decreases the concentration of the peptone inhibitors by dilution, active enzyme is liberated from the pepsin-peptone complex and additional digestion occurs. The significance of these concepts will be enlarged upon later in this paper.

The rate of digestion, as heretofore mentioned, is directly proportional to the rate of concentration of the active substrate (21) which is steadily diminishing during digestion. This, together with the fact that the enzyme is being progressively inactivated during digestion, does not allow for the expression of a direct linear relationship between the enzyme concentration and the amount of protein digested under practical test conditions. Northrop (20) has found the kinetics of the reaction to conform to the following equation:

$$\frac{A \operatorname{Log} \dfrac{A}{A - x} - x}{ET} = K$$

A = Substrate concentration
x = Quantity of substrate converted
E = Total enzyme concentration (active and inactive)
T = Time.

Under the experimental conditions of our test, the amount of protein digested is directly proportional to the logarithm of the total pepsin concentration (Figure 2).

Protein is an amphoteric substance and is present in the form of kations in a solution acid to its isoelectric point (22). Pepsin acts only on pepsin kations. (The effect of hydrogen ion concentration on pepsin digestion is purely on the substrate and *not* on the enzyme [23].)

The percentage of protein present in its kationic form increases as the pH of the solvent decreases until 100 per cent is in the kationic form. Thereafter, further decrement in pH will reduce the number of protein kations by the common ion effect. An increase in the active kationic substrate, brought about by pH changes, augments the rate of digestion according to the Law of Mass Action. Since the pH at which all protein is in its kationic form varies from protein to protein, the optimum digestion pH of proteins differs. Peptic digestion of mucosa mimics egg albumen in that both have their optimum digestion pH in the region of 1 (24). The digestion of mucosa may therefore be compared to egg albumen.

[2] Northrop uses the word "peptones" loosely to include all those breakdown products of protein which combine with pepsin. We have retained his definition. We have also included gastric inhibitory substances with peptones, since they certainly react like them and for the purposes of this paper may be considered identical.

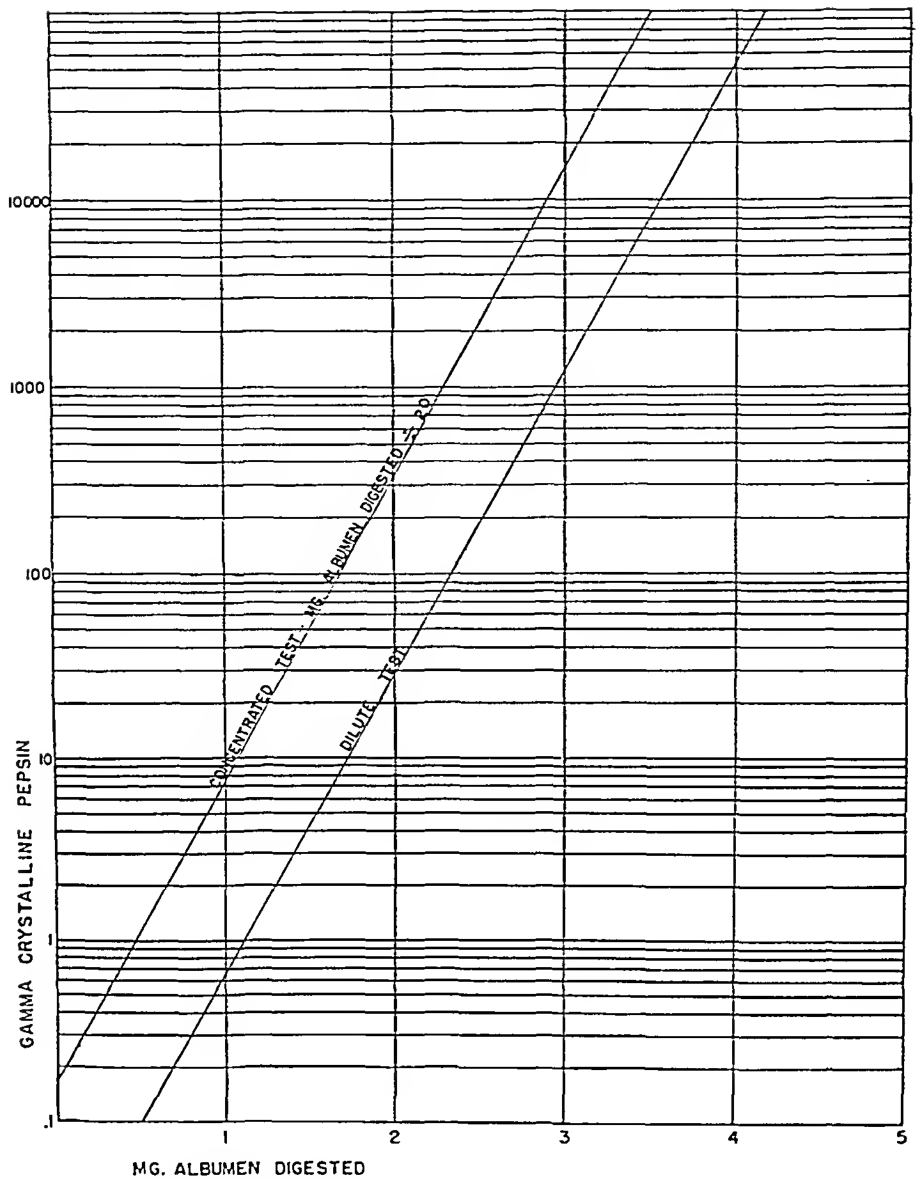

FIG. 2. THE NUMBER OF MILLIGRAMS ALBUMEN DIGESTED IS PLOTTED AGAINST GAMMA OF CRYSTALLINE PEPSIN ON SEMILOGARITHMIC PAPER FOR BOTH THE DILUTE AND THE CONCENTRATED TESTS

In the concentrated test, 2½ × the enzyme concentration produces 20 × the amount of albumen digested.

APPLICATION OF ENZYME KINETICS TO THE STUDY OF GASTRIC JUICE

In gastric juice, the estimation of pepsin activity is complicated by the presence of inhibitory substances analogous to peptones (25). These substances, formerly called anti-enzymes (26), have been thought to play an active role in protecting against autodigestion (26, 27). Langenskiöld (28) indicated that these substances were probably peptones. It may be that the inhibitors of the gastric juice are peptones formed by the action of pepsin on proteins derived from food, mucosal cells, and secreted protein (mucin and plasma protein).

Dilution of gastric juice, under any conditions, by decreasing the concentration of inhibitory sub-

stances (peptones), allows digestion to continue unimpeded. By using highly diluted juice one can assay the total quantity of pepsin present unaffected by the naturally occurring inhibitory substances. Conversely, when one uses low dilutions, one assays only that fraction of pepsin which is uncombined with the naturally occurring inhibitory substances, which we have called the Active Fraction. Bucher, Grossman and Ivy (29) have found that separation is usually complete at 1:100 dilution so that no further augmentation in pepsin activity occurs on continued dilution.

The preceding discussion poses the corollary if tests were performed on undiluted gastric juice, only that fraction of pepsin uncombined with inhibitor would be measured. Even when the gastric juice is mixed with concentrated substrate, however, dilution is unavoidable. In order to correct for this dilution, a curve correlating low dilution of gastric juice with quantity of substrate decomposed has been constructed (Figure 3). (This relationship is observed only in low dilutions.) This establishes that one can interpolate to the undiluted state in order to determine the amount of pepsin in gastric juice that remains unaffected by the inhibitory substances present (30).

We have assayed Total Pepsin Activity at optimum pH utilizing high dilutions of gastric juice (16). At the same pH we have assayed that amount which remains uninhibited (active fraction) by employing low dilutions of gastric juice and interpolating to the undiluted state. The per cent of pepsin inhibition may be thus derived.

Alterations in pepsin activity of this active fraction, produced by variation of pH from the optimum, are depicted graphically (Figure 4). It is evident that the acidity of different gastric juices varies significantly from this optimum. Hence the activity of the pepsin will vary and can be computed by reference to the figure. This pep-

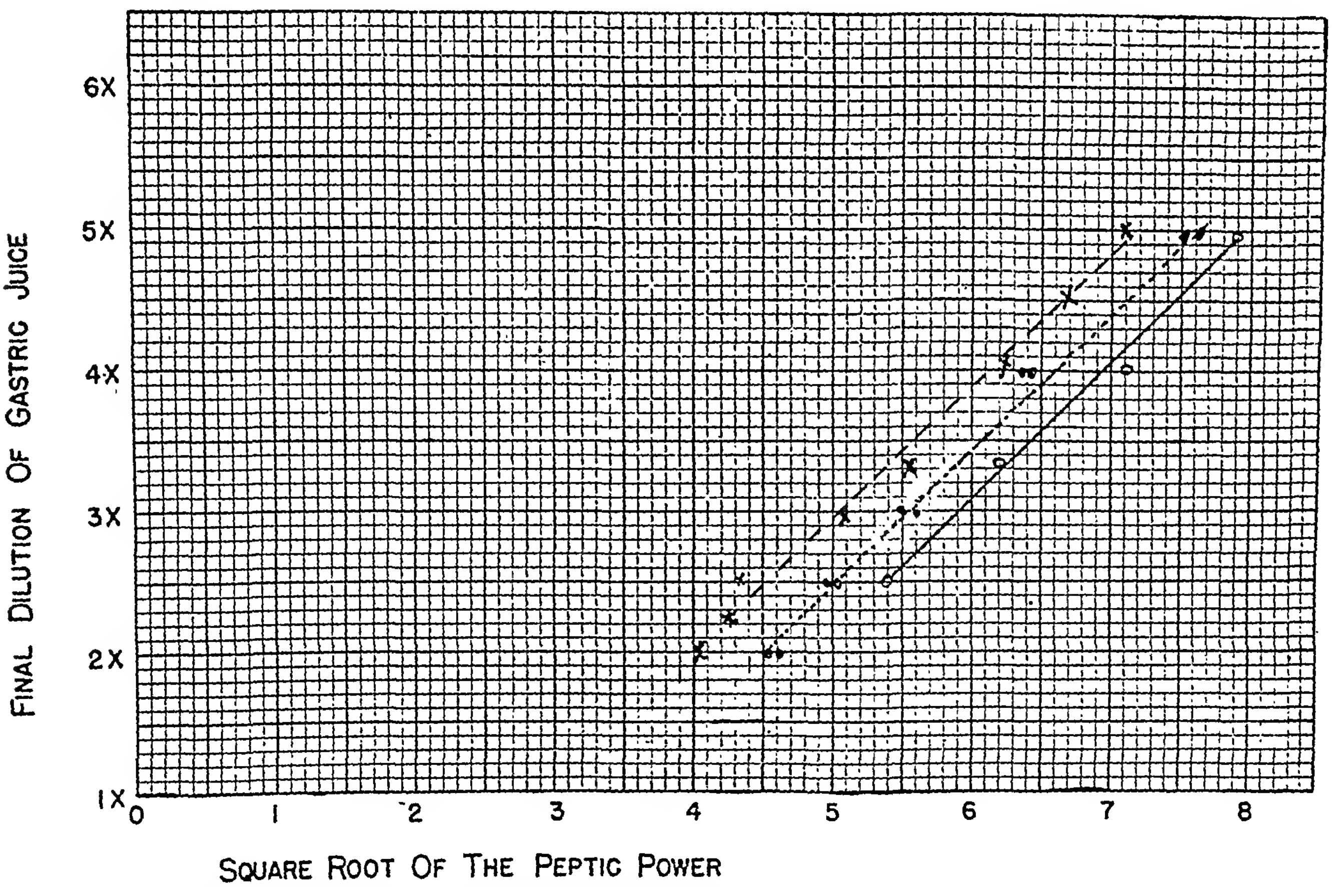

FIG. 3. SHOWS THE RELATIONSHIP OF DILUTION (3 DIFFERENT GASTRIC JUICES) TO THE SQUARE ROOT OF THE MILLIGRAMS OF ALBUMEN DIGESTED PER ML. OF GASTRIC JUICE

1x is point of no dilution. The represented relationship is 1:1 so that each increase of 1x in dilution increases the square of the peptic power 1.

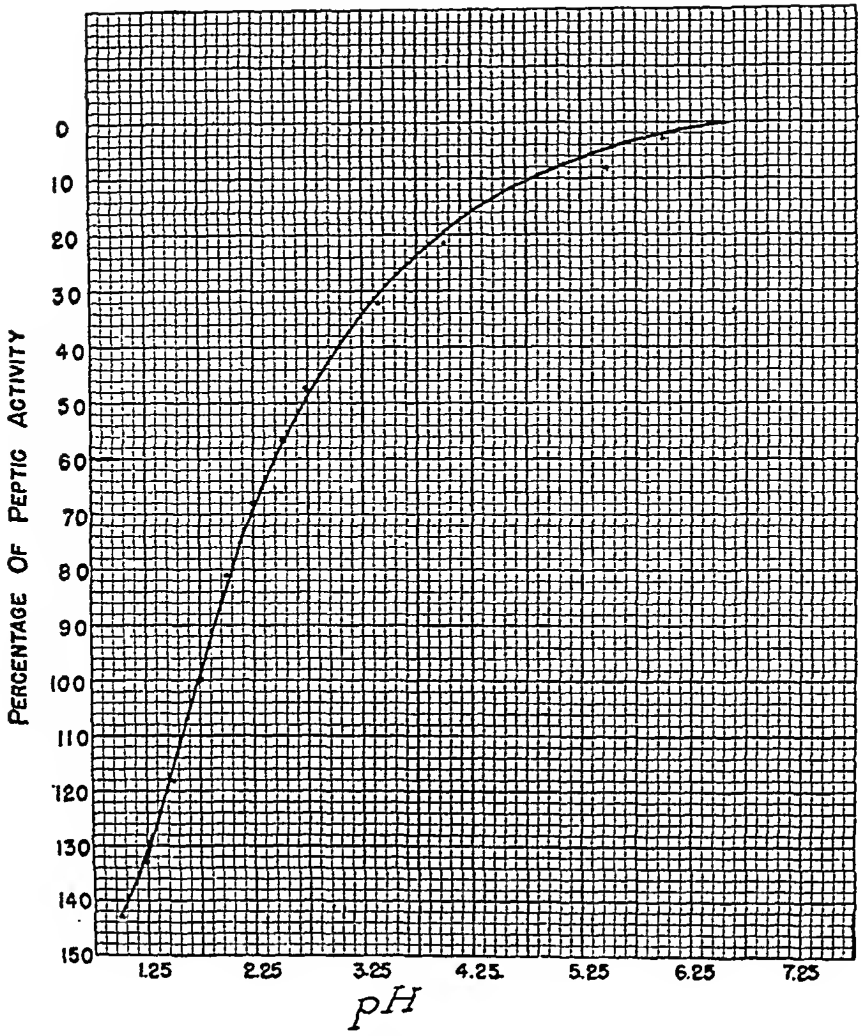

FIG. 4. DIGESTED PROTEIN, REPRESENTED AS THE PERCENTAGE OF PROTEIN DIGESTED AT pH 1.75 (100 PER CENT), IS PLOTTED AGAINST THE HYDROGEN ION CONCENTRATION OF THE DIGESTION MIXTURE EXPRESSED IN pH UNITS

sin activity, corrected for the effect of inhibitor substances and of pH, we have designated Resting Peptic Activity. It is a measure of the pepsin activity which the gastric and duodenal mucosa are resisting in the basal secretory state.

To reiterate, total pepsin activity is merely an index of the secretory activity of the pepsinogenic cells of the gastric mucosa, but does not indicate the true digestive activity of the gastric juice as modified by the inhibitor substances and hydrogen ion concentration.

Since inhibitory substances and pH play such a significant role in the kinetics of the pepsin reaction, it would seem important to know how much pepsin is inhibited via each mechanism in normal and ulcer patients. Up to the present, there has been no information in the literature concerning this.

MATERIALS AND METHODS

All determinations were performed 4 to 8 hours after collection of the gastric juice. pH was determined with a glass electrode.

The methods for pepsin measurements have been described in detail elsewhere (16, 30). A brief outline is given to provide the reader with some conception both of the technic and the derivation of the units.

By precipitating various concentrations of albumen with sulfosalicylic acid under conditions of dilution and hydrogen ion concentration similar to those carried out in

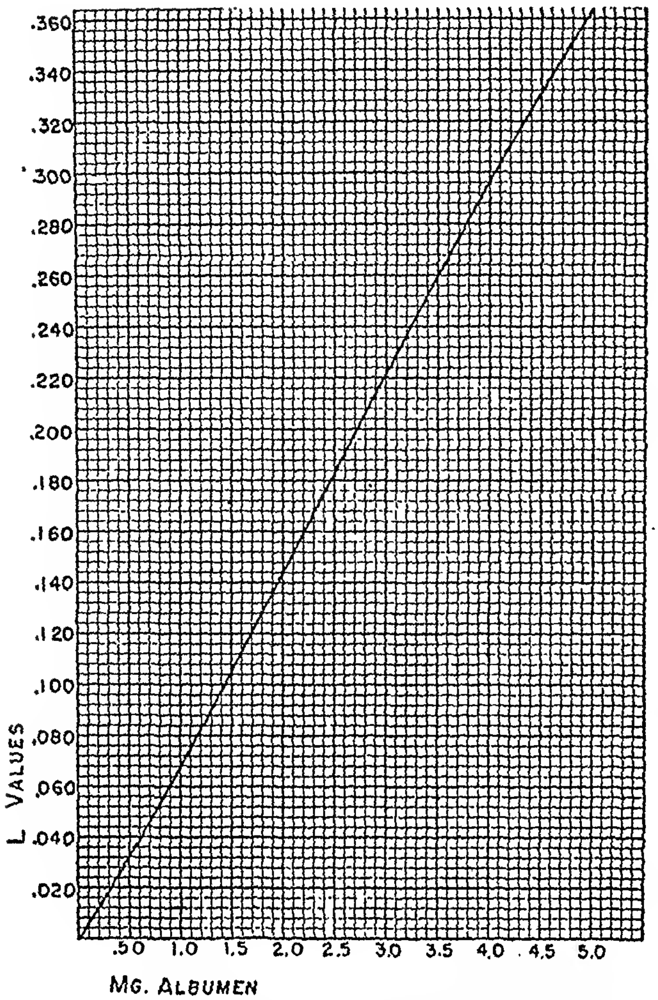

FIG. 5. SHOWS THE RELATIONSHIP OF MGM. ALBUMEN PRECIPITATED BY SULFOSALYCYLIC ACID TO OPTICAL DENSITY EXPRESSED AS L-VALUES

the test, and recording the turbidity in the Evelyn Colorimeter, a nomogram was constructed which related optical density, or L values (Figure 5) to mgm. of albumen present and enabled one to determine by difference the amount of albumen digested.

Although the pepsin determinations in both tests have been standardized against crystalline pepsin,[3] it has been deemed desirable to use relative units, Pepsin Equivalents, instead, because there is no uniformity of activity among the various crystalline preparations of pepsin (31). Our pepsin equivalent unit is equal to 0.076 gamma of crystalline pepsin. (About 50 per cent of this preparation is magnesium sulfate.)

Dilute test—assay of total pepsin

Five mgm. of egg albumen is incubated for 30 minutes at 37° C. with diluted gastric juice so that the final dilution is 1/563. The mgm. albumen digested are determined by precipitating with sulfosalicylic acid after inactivating the pepsin with sodium hydroxide. Units proportional to actual quantities of crystalline pepsin are obtained by expressing the results as the antilogarithm of the mgm. albumen digested. By multiplying the dilution factor, the final result, total pepsin, is designated in pepsin equivalents per ml. of gastric juice.

Concentrated test—assay of active pepsin

Undiluted gastric juice is mixed with concentrated albumen solution and hydrochloric acid solution so that the final dilution of the gastric juice is in the order of ¼. Digestion is carried out at 37° C. for 30 minutes. A duplicate sample is used to determine the pH of the digestion mixture. By use of Figure 4, the amount of albumen which would have been digested at pH 1.75 (the digestion pH of the dilute test) is determined.

Because the lines represented in Figure 3 have the same slope, it is possible to extrapolate the results obtained at ¼ dilution to zero dilution.

After adjusting for the differences in kinetics of the two tests, results are again expressed in pepsin equivalents as before. The units thus derived give values for the active pepsin which are comparable to those for total pepsin. Since the units are comparable the percentage of pepsin inhibited is easily derived from the figures for total pepsin and active pepsin.

Resting peptic activity

Since the active pepsin fraction is expressed as the activity at pH 1.75, it will not represent the activity of the gastric juice at its own pH occurring *in vivo*.

After obtaining the pH of the gastric juice, the active pepsin fraction is corrected to the activity at that pH by reference to Figure 4.

This value shows the summation of the effect of inhibitory substances and pH characteristic of a specimen of gastric juice collected during a basal secretory state. It should be remembered that this is an artificial comparison because the effect of pH is on the substrate and not on the enzyme. It really represents the activity which would result if the enzyme concentration were reduced at a constant pH.

Peptic activity in human subjects

A total of 132 fasting specimens of human gastric juice was obtained from the Gastrointestinal Clinic of the University of Chicago Clinics[4] and the determinations earlier described were made. Out of this number we selected juice from every case of proved duodenal ulcer, totaling 30 cases. All patients had x-ray evidence of ulcer.

Twenty-one fasting specimens of gastric juice were collected from 21 of the students, interns, and resident staff of the Clinics. The criteria for normality were absence of past or present gastrointestinal disease or symptoms.

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[3] The crystalline pepsin used in this study was obtained from the Plaut Research Laboratories, Bloomfield, N. J.

[4] Through the courtesy of Dr. Walter L. Palmer.

TABLE 1

Pepsin activity of gastric juice
30 duodenal ulcer subjects
21 normal subjects

Total pepsin activity. Pepsin equivalents per ml.		Active pepsin fraction. Pepsin equivalents per ml.		pH of gastric juice		Resting peptic activity. Pepsin equivalents per ml.	
Normal	Ulcer	Normal	Ulcer	Normal	Ulcer	Normal	Ulcer
2,500	2,500	2.6	1.2	5.12	1.35	0.5	1.5
3,400	3,900	1.7	1.0	3.25	2.55	0.8	0.7
4,300	3,000	0.6	1.1	1.85	2.15	0.6	0.9
4,300	5,200	4.4	1.2	1.50	1.45	5.9	1.5
313	2,700	1.1	1.3	4.90	1.10	0.8	1.9
7,500	3,600	2.2	1.7	1.50	2.20	2.8	1.1
188	2,500	0.6	1.3	7.45	1.40	0	1.6
1,900	6,200	1.7	1.6	4.55	1.50	0.6	2.1
6,100	940	1.0	1.8	1.80	2.10	0.9	1.3
250	4,900	0	1.3	6.45	2.0	0	1.1
6,100	2,500	1.5	1.0	1.50	1.50	1.9	1.1
8,600	6,200	1.3	1.2	1.60	2.30	1.5	0.8
4,300	8,700	1.2	1.4	1.80	1.50	1.1	1.7
3,900	5,400	1.1	1.9	1.55	1.45	1.3	2.5
3,900	4,800	1.1	1.0	2.55	3.10	0.7	0.6
3,600	11,200	1.2	1.8	2.70	1.50	0.8	2.2
2,800	3,600	0.9	1.0	1.55	1.40	1.0	1.1
6,100	2,500	0.8	1.0	2.25	1.50	0.7	1.1
7,500	4,900	2.8	1.3	1.40	1.30	4.2	1.7
1,600	4,100	1.1	1.0	3.55	1.50	0.6	1.1
2,800	1,300	5.6	1.9	3.70	1.80	1.1	1.8
	1,000		1.3		1.30		1.8
	5,200		1.6		1.55		1.9
	3,600		0.9		1.50		1.0
	4,300		1.1		1.65		1.1
	3,400		0.6		1.40		0.7
	3,900		1.3		1.50		1.5
	4,900		1.4		1.25		2.0
	3,000		1.1		1.60		1.1
	2,500		0.9		1.50		1.0

RESULTS

The results of the pH determinations on the ulcer patients and on the normal human beings are depicted in Table I. One may note that 28 out of 30 ulcer patients, or 92 per cent, had pH values below 2.4, whereas only 11 out of 21 of the normal group, or 52 per cent had high free acid values in the corresponding pH range (Figure 6). This is in fair agreement with what has been described in the literature by other investigators (1, 2).

An examination of the peptic activity of the gastric juices of our human subjects, as illustrated in Table I, reveals the following:

Total pepsin activity. It is notable that there is wide individual variation in the total pepsin activity (or pepsin content) among both the normal and the ulcer groups. Despite this variation, statistical comparison of the two groups as a whole reveals no significant difference.

Active pepsin fraction. Here one may see that the amount of inhibitory substances present is of a huge magnitude for all individuals in both groups, for the actual amount of active pepsin present is very small. Notwithstanding the wide individual variation in total pepsin activity existing among individuals in each group, the active pepsin fraction does not exhibit the same trend, but rather there is close uniformity in the quantity

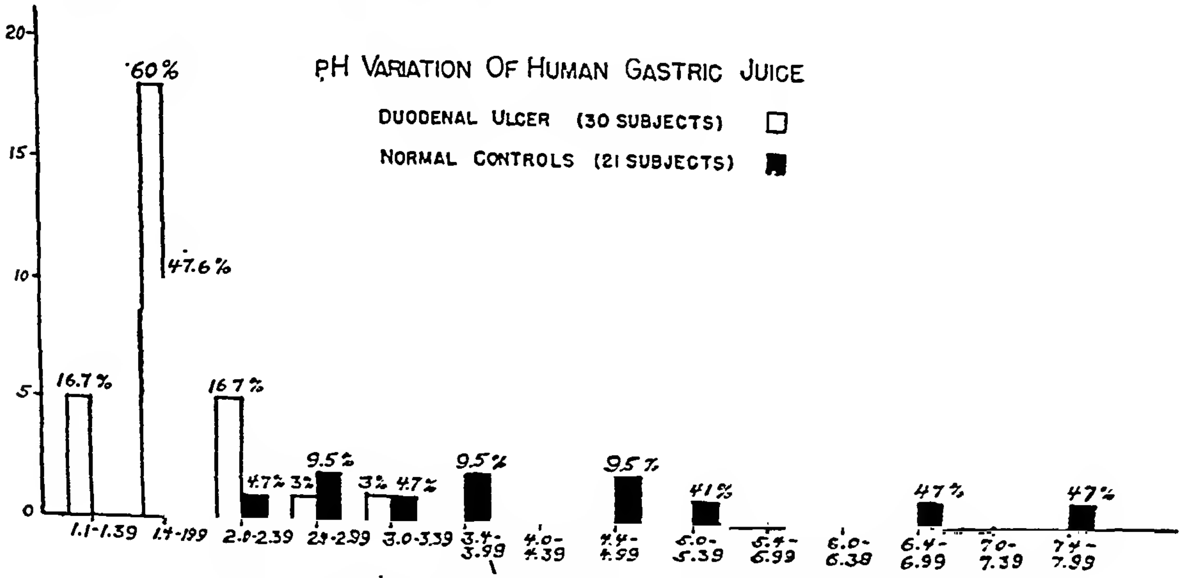

FIG. 6. SHOWS THE DISTRIBUTION OF CASES ACCORDING TO THEIR GASTRIC ACIDITY IN pH UNITS

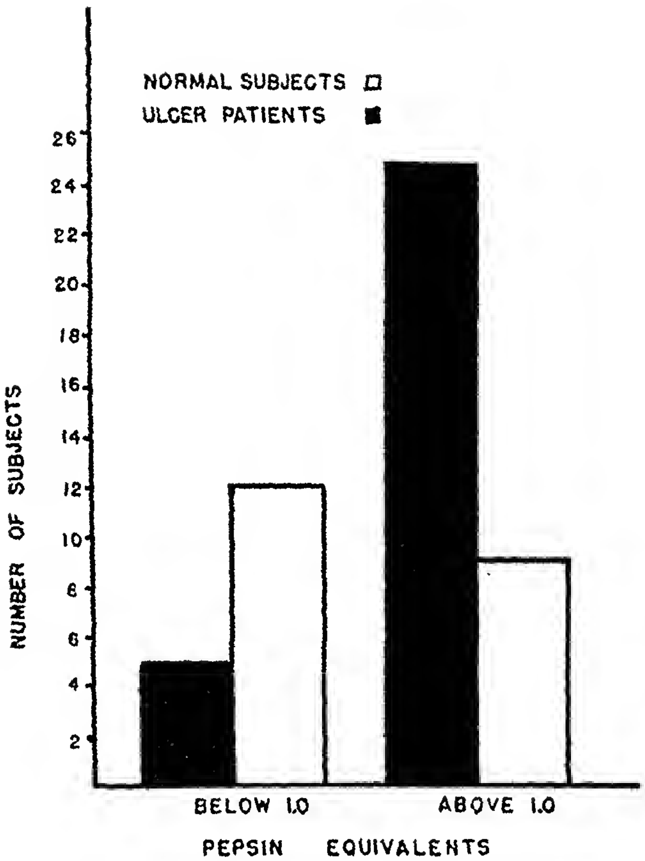

FIG. 7. THE NUMBER OF CASES HAVING RESTING PEPTIC ACTIVITY ABOVE AND BELOW THE VALUE OF 1 IS ILLUSTRATED

of pepsin in both groups. Again, statistical comparison between the two groups shows no significant difference.

Per cent pepsin inhibition. This illustrates again the enormous amount of inhibition present and the uniformity of this phenomenon in all the individuals studied. In all but one case, more than 99 per cent of the total pepsin is inhibited, and in most, there is 99.9 + per cent inhibition.

The percentage of the total pepsin which is active in the presence of the pepsin inhibitory substances of the gastric juice is exceedingly small. With the crystalline pepsin used by us, the quantity of active pepsin present ranges approximately from 0.05 gamma to 0.15 gamma per ml., as compared with an average figure of approximately 270.0 gamma per ml. of total pepsin in both groups.

Resting peptic activity. This estimation of the actual *in situ* digestive activity of the gastric juice, expressed in pepsin equivalents, is also contained in the table.

It is here that one may note the effect of pH of the gastric juice on the active pepsin fraction. Precursory inspection may not reveal a striking change because of 3 abnormally high results in the normal group. However, the majority of ulcer cases, 25 out of 30, have pepsin activities of 1.0 pepsin equivalents or above, whereas less than half, 9 out of 21 normal subjects, have like values. This is pictured in Figure 7. That this result is significant has been proved by the Chi Square Method of Statistical Analysis ($\chi^2 = 9.1$).

DISCUSSION

From the evidence presented, it is manifest that the major differences between the two groups lie in the pH and resting peptic activity. The active pepsin fraction, percentage of inhibition, and total pepsin concentration are almost identical in the two groups.

It is noteworthy that our findings of similar total pepsin concentrations in the normals and in ulcer patients are at variance with the reports of other investigators (10, 11, 12). A criticism that might be made of our findings is the relative smallness of the series. However, the number of cases studied is statistically adequate. We feel that the findings of others quoted (10, 12) may be open to criticism on the basis of error inherent in their method of pepsin determination, as mentioned previously. Vanzant *et al* (10) and Mullins and Flood (11) both used low dilutions of gastric juice, which resulted in the assay, not of total pepsin, but of a variable mixture of pepsin and pepsin-inhibitor complex. Both also obtained their gastric specimens after test meal stimulation. There is some indication that the test employed by Osterberg *et al* was subject to error because of insufficient buffering of their digestion mixture. In our experience, even strong buffers will not maintain a constant digestion pH when mixed with equal volumes of gastric juice. We have occasionally observed unusually high fasting values of total pepsin in ulcer patients, but the group as a whole does not vary significantly from the normals.

The observation that 99 + per cent of the pepsin is inhibited might seem incredible at first. Examination of Figure 1 relating quantity of albumen digested per unit time reveals a rapid initial rate becoming negligible after 30 minutes. Inasmuch as the rate of digestion is directly proportional to the concentration of active enzyme and since the

rate of digestion becomes practically nil, it follows that there must be an extremely small amount of active enzyme present. The pepsin in resting gastric contents is constantly acting on protein substrate (cells, mucin, and protein secreted), and one might expect that it is almost completely inhibited for the same reason. This may well be one of the reasons that the stomach does not digest itself. Formerly, one remarked at the amazing ability of the stomach and duodenum to resist digestion. Now it can be seen that these organs actually have very little to resist and that instead of being resistant, they are probably quite susceptible. The inability of these organs to resist digestion is indicated by those animal experiments in which the feeding or irrigation of bowel with active pepsin-hydrochloric acid mixture quite uniformally and rapidly produced ulceration (13, 14, 15). The rapidity with which apparently normal humans develop perforation and bleeding under certain emotional stresses (32) also, the rapidity with which peptic ulcers sometimes develop after burns (33) would seem to indicate that the mucosa is not resistant to attack by active gastric juice. Herein also may lie the explanation of how ulcers, produced by the ingestion of acid in the experimental animal (4), may result from dilution of inhibitor substances and also further enhancement of the enzyme action by the increased hydrogen ion concentration. Hypersecretion also, by continually diluting the inhibitor substances, might sustain active pepsin at relatively high levels.

The extremely low values of the active pepsin fraction would tend to support the contention that very little pepsin is necessary to produce mucosal ulceration via the digestion mechanisms. Despite the high percentage of observed inhibition, the pepsin is not really destroyed, and is readily made available for digestion by the process of dilution. There is a huge pepsin reserve; the digestive power of gastric juice can be increased 5,000 times by simple dilution. As mentioned in the section on kinetics, even the addition of a sizable increment of pepsin to a pepsin solution with a high concentration of inhibitor will produce little increase in digestive action. For this reason, the actual digestibility of gastric juice does not vary appreciably with excessive increases in pepsin content. Driver (14) found that doubling the pepsin concentration did not alter the extent or frequency of ulceration observed when perfusing isolated loops of small intestine. His perfusion was carried out at the rate of 2 ml. per minute through a loop of bowel that would hold many times that volume. The perfusion was certainly not rapid enough to wash out the inhibitors that formed during the process of peptic digestion, and thus one may conclude that the active pepsin fraction was not significantly elevated. In the discussion on kinetics, it was stated that the amount of albumen digested is not in linear proportion to the pepsin concentration. Therefore, increasing the concentration of pepsin should not change the ulcerating power markedly either in the dog experiments of Driver (14) or the frog experiments of Dragstedt (5). Matthes (34) who was one of the first to irrigate loops of bowel in dogs with pepsin-hydrochloric acid mixtures and gastric juice, concluded that peptones found in gastric juice combined with the free acid and in this way reduced its ulcerating ability. Langenskiöld (28) came to similar conclusions. Their work, however, really lends excellent support to the thesis that ulcers are the product of peptic digestion since the peptones more effectively combine with the pepsin.

Even though large changes in the pepsin concentration do not produce appreciable increase in pepsin activity, small changes in hydrogen ion concentration will produce marked changes in digestive action (Figure 4). Hence this mechanism is by far the most important in controlling the digestive action *in vivo*. It can therefore be established that the pH is of greater importance in controlling resting peptic activity than the total pepsin content of the gastric juice. On comparison of the resting peptic activity of the normals with those of ulcer patients, one sees no clear-cut division between the two groups. Some normals, drawn from medical students and interns, had high gastric acidity and therefore high resting peptic activity. In some cases these individuals said they were hungry because they had missed their accustomed breakfast; others were apprehensive and had difficulty swallowing the tube. The studies of Wolf and Wolff (35) on Tom, a man with a stricture of the esophagus and a large gastric fistula, have shown that emotional stimuli such as resentment and anxiety will increase hydrochloric

acid production tremendously. Also, hunger will produce copious secretion of hydrochloric acid and pepsin. We have not excluded these normals from our series, however, since the ulcer patients have probably experienced the same stimuli. Many of both groups, therefore, were in a secretory state rather than a basal state. True resting contents are well-nigh impossible to obtain under the usual clinical conditions (36). One may always expect some high values in the group. The best method of comparing these two groups is by statistical means. When such a comparison is made the results have been shown to be highly significant.

A better method of investigation than ours for obtaining basal secretory levels would be gastric aspiration during sleep. Winkelstein (37) and others (38, 39) have pointed out that the free hydrochloric acid falls to zero or very low in normal patients during sleep; yet, in sharp contrast, increased values are found in ulcer patients. Such a study, however, necessitates hospitalization.

These present observations on gastric juice while highly suggestive do not prove that ulcer development is a function of the resting peptic activity. One must prove that the ability of pepsin solution to digest a given protein substrate is the same as its ability to produce experimental ulcers.

Experiments (24) have been conducted wherein the mucosal and serosal surfaces of dogs' intestines were irrigated with hydrochloric acid-pepsin solutions adjusted so that the pH of one pepsin mixture was kept at 1.50 and the other at 2.15. This made the peptic activity of the pH 2.15 mixture 60 per cent of that of the pH 1.50 preparation (Figure 4). The activity of the former solution was doubled, however, by increasing its temperature 10° C. (van't Hoff's Law) so that its final activity was 20 per cent greater than that of the more acid solution. (The irrigation temperature was 30° C. for the pH 1.5 solution and 40° C. for the other.) By this means the pepsin activity of the less acid solution was the greater. Ulcers formed more readily and were more extensive both on the mucosal and serosal surfaces of the bowel with the low acid-high peptic activity mixture. Bowel irrigated with hydrochloric acid alone (pH 1.5 and at 40° C.) exhibited no ulceration. When conditions other than temperature variations were sought to reduce peptic activity, it was found that 2 per cent solution of neopeptone ("Difco") reduced the amount of albumen digested by 50 per cent. Bowel was irrigated with two acid pepsin solutions both at pH 1.5 but one containing 2 per cent neopeptone. Neopeptone protected against the development of ulcers. It will be remembered that Matthes (34) had concluded that peptone in gastric juice by reducing the free acid had lessened its ulcerating effect. In the experiment reported, acid was added to the solution containing neopeptone to make the pHs identical, yet the solution containing neopeptone did not readily form ulcers. Such exclusive experiments would tend to indicate that peptic activity plays a major role in the production of ulcers.

CONCLUSIONS

Enzyme kinetics, together with quantitative methods for the determination of total pepsin concentration and inhibitor substances, have been applied in a physiological study of the products of gastric secretion in 31 proved ulcer patients and 21 normal subjects.

The resting peptic activity was found to be significantly greater in the ulcer group. This increase was dependent upon the higher acidity of the gastric juice in the ulcer patients, for there was no increase in total gastric pepsin in this group. The reason that an increase in total pepsin content of gastric juice does not significantly alter peptic activity is that pepsin inhibitor substances are present.

The inhibitor substances present in the gastric juice produce more than 99 per cent inhibition of pepsin in both the normal and the ulcer groups. This, in conjunction with the probable low basal levels of free hydrochloric acid in normal patients, prevents autodigestion.

It would appear that ulcers are really the product of peptic digestion, and that the pH of the gastric juice is by far the most important regulator of the peptic activity, even to the exclusion of pepsin concentration.

It is believed that strict application of the laws of enzyme chemistry has elucidated some discrepancies which have resulted from the inadequate qualitative experiments of the past on gastric digestion.

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BIBLIOGRAPHY

1. Sagal, Z., Marks, J. A., and Kantor, J. L., The clinical significance of gastric acidity. A study of 6679 cases with digestive symptoms. Ann. Int. Med., 1933, 7, 76.
2. Vanzant, F. R., Alvarez, W. C., Berkson, J., and Eusterman, G. B., Changes in gastric acidity in peptic ulcer, cholecystitis, and other diseases (analyzed with the help of a new and accurate technique). Arch. Int. Med., 52, 616, 1933.
3. Dragstedt, L. R., Pathogenesis of gastroduodenal ulcer. Arch. Surg., 1942, 44, 438.
4. Mann, F. C., and Bollman, J. L., Experimentally produced peptic ulcers; development and treatment. J. A. M. A., 1932, 99, 1576.
5. Dragstedt, L. R., Acid Ulcers. Surg. Gyn. & Obstet., 1936, 62, 118.
6. Vineberg, A. M., and Babkin, B. P., Histamine and pilocarpine in relation to the gastric secretion. Am. J. Physiol., 1931, 97, 69.
7. Gillman, A., and Cowgill, G. R., The effect of histamine upon the secretion of gastric pepsin. Am. J. Physiol., 1931, 97, 124.
8. Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., The experimental production of gastric and duodenal ulcers in laboratory animals by the intramuscular injection of histamine in beeswax. Surg. Gynec. & Obstet., 1942, 75, 170.
9. McHardy, G., and Browne, D. C., Duodenal ulcer developing in man following "histamine desensitization." Gastroenterology, 1944, 2, 345.
10. Vanzant, F. R., Osterberg, A. E., Alvarez, W. C., and Rivers, A. B., Studies of gastric pepsin. II. Secretion of pepsin in cases of duodenal ulcer and pseudo-ulcer. J. Clin. Invest., 1933, 12, 557.
11. Mullins, C. R., and Flood C. A., A study of gastric pepsin in various diseases. J. Clin. Invest., 1935, 14, 793.
12. Barowsky, H., Upham, R., Dotti, L. B., and Kleiner, I. S., Clinical significance of concentration of pepsin in gastric juice. Rev. Gastroenterol., 1943, 10, 201.
13. Schiffrin, M. J., and Warren, A. A., Factors (especially gastric acidity and pepsin) concerned in the production of experimental ulceration of the gastrointestinal tract of cats. Am. J. Digest. Dis., 1942, 9, 205.
14. Driver, R. L., Chappell, R. H., and Carmichael, E. B., Effect of concentration of pepsin and differential susceptibility of jejunal segments in experimental ulcers in dogs. Am. J. Digest. Dis., 1945, 12, 166.
15. Matzner, M. J., Windwer, C., Sobel, A. E., and Polayes, S. H., Role of pepsin in experimental production of gastric ulcer in rats. Proc. Soc. Exper. Biol. & Med., 1936, 34, 243.
16. Le Veen, H. H., Total peptic activity of gastric juice, a method of determination. Proc. Soc. Exper. Biol. & Med., 1946, 63, 254.
17. Waldschmidt-Leitz, E., Enzyme Action and Properties. J. Wiley & Sons, N. Y. 1929, p. 134.
18. Tiselius A., and Eriksson-Quensel, I., Some observations on peptic digestion of egg albumen. Biochem. J., 1939, 33, 1752.
19. Bergmann, M., and Fruton, J. S., The specificity of proteinases. Advances Enzymol., 1941, 1, 63.
20. Northrop, J. H., The effect of concentration of enzyme—on the rate of digestion of proteins by pepsin. J. Gen. Physiol., 1920, 2, 471.
21. Northrop, J. H., The influence of substrate concentration on the rate of hydrolysis of proteins by pepsin. J. Gen. Physiol., 1920, 2, 595.
22. Hitchock, D. L., In Schmidt's "The Chemistry of the Amino Acids and Proteins." 2nd edition. Charles C. Thomas Springfield, Ill., 1945, p. 596.
23. Ringer, W. E., Quoted by Northrop, J. H., Combination of enzyme and substrate. II. Effect of hydrogen ion concentration. J. Gen. Physiol., 1920, 2, 113.
24. Le Veen, H. H., Chemical, Physiological, and Pathological observations on the role of pepsin and hydrochloric acid in the production of experimental ulcers. Gastroenterology, 1947, 8, 648.
25. Nirenstein, E., and Schiff, A., IV. Über die pepsinbestimung nach mette und die nothwendigkeit ihrer modification für klinische zwecke. Berlin. Klin. Wchnschr., 1903, 40, 268.
26. Hensel—Quoted by Komarov, S. A., Inactivation of pepsin and its relation to peptic ulcer. Rev. Gastroenterol., 1942, 9, 165.
27. Blum, L., and Fuld, E., Über das Vorkommen eines Antipepsin in Magenshaft. Ztschr. F. Klin. Med., 1906, 58, 505.
28. Langenskiöld, F., Über die widerstandsfähigkeit einiger lebender Gewebe gegen die Einwirkung eiweisspaltender Enzyme. Skand. Arch. F. Physiol., 1914, 31, 1.
29. Bucher, G. R., Grossman, M. I., and Ivy, A. C., A pepsin method: The role of dilution in the determination of peptic activity. Gastroenterology, 1945, 5, 501.
30. Le Veen, H. H., Active pepsin fraction, resting peptic activity, and percentage of pepsin inhibition gastric juice. Proc. Soc. Exper. Biol. & Med., 1946, 63, 259.
31. Wiss, O., Investigations into proteases (1) amino acids, hydrocyanic acid, pyrophosphates as effectors of pepsin. Helvetica Chimica Acta, 1946, 29, 237.
32. Davies, D. T., and Wilson, A. T. M., Personal and clinical history in hematemesis and perforation. Lancet, 1939, 2, 723.

33. Harkins, H. N., Acute ulcer of the duodenum (Curling's Ulcer), as a complication of burns; relation to sepsis. Surgery, 1938, 3, 608.
34. Matthes, Max, Untersuchung über die Pathogenese des Ulcus rotundum ventriculi und über den Einfluss von Verdauungsenzym auf lebendes und todtes Gewebe. Beitr. Path. Anat. u. F. Allg. Path., 1893, 13, 309.
35. Wolf, S., and Wolff, H. G., Human Gastric Function. Oxford University Press, N. Y. 1943, p. 109.
36. Bloomfield, A. L., Chen, C. K., and French, L. R., Basal gastric secretion as a clinical test of gastric function with special reference to peptic ulcer. J. Clin. Invest., 1940, 19, 863.
37. Winkelstein, A., One hundred and sixty-nine studies in gastric secretion during the night. Am. J. Digest. Dis. & Nutrition, 1935, 1, 778.
38. Henning, N., and Norpoth, L., Die Magensekretion während des Schlafes. Deut. Arch. F. Klin. Med., 1932, 172, 558.
39. Mittelmann, B., and Wolff, H. G., Emotions and gastroduodenal function; experimental studies on patients with gastritis, duodenitis and peptic ulcer. Psychosom. Med., 1942, 4, 5.

THE TOLERANCE FOR POTASSIUM IN SEVERE RENAL INSUFFICIENCY: A STUDY OF TEN CASES

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The concentration of potassium in the serum of a patient who has developed severe renal insufficiency may be normal, increased, or diminished, and no satisfactory explanation for these widely varied concentrations has been advanced. Naturally the finding of an increased content of potassium in the serum of a nephritic patient suggests caution in the administration of potassium salts. On the other hand there are certain undernourished patients who have nephritis and are suffering from lack of potassium and therefore require an increased intake. It is also recognized that many patients who have renal edema can tolerate a considerable daily dose of potassium salts over a period of months without evidences of toxic effects. It is therefore obvious that there are other factors, in addition to renal retention, which play a role in the distribution of the potassium ion in uremic patients.

Observations of potassium tolerance, both in normal subjects and in patients who had adrenal insufficiency, have been made by Zwemer and Truszkowski (1), Wilder and his co-workers (2), and Jaffe and Byron (3). The effects of a small and of a considerable dose of potassium salts were studied by Greene, Levine and Johnston (4), and by Winkler and his associates (5) in renal disease and by Thomson (6) and Sharpey-Schafer (7) in cardiac disease. The doses employed by Winkler and Sharpey-Schafer were frequently large and sometimes proved impractical and even dangerous. We therefore gave normal subjects and patients who had renal disease a uniform and smaller dose of 5.0 grams of potassium bicarbonate by mouth and were gratified to find that this amount could usually be tolerated and also revealed information as to the individual's tolerance. Our procedures and results in normal subjects have already been reported (8). The present communication deals with the results of the same procedures carried out in 10 patients who had definite renal insufficiency. Distinct alterations in potassium tolerance were observed in all of the 10 patients and form the basis of this paper.

SELECTION OF PATIENTS

The chief consideration in the selection of our patients was demonstrable renal insufficiency. Eight of the 10 patients were deliberately chosen because the degree of renal incompetence was marked and we desired particularly to learn the tolerance for potassium of patients who had a minimal amount of functioning renal tissue. The degree of renal insufficiency of these 8 patients was indicated by the increase in the concentrations of urea and creatinine in the blood from 147 to 340 mgm. and from 5.6 to 19.2 mgm. per 100 ml., respectively. The diagnosis in 6 patients was chronic glomerulonephritis; in the 4 remaining, respectively, it was subacute glomerulonephritis, chronic pyelonephritis, bilateral congenital hydronephrosis, and multiple myeloma with chronic diffuse nephritis. Death occurred in 9 of our patients in 2 weeks to 8 months after the test was done. One patient, J. W., with the least degree of renal incompetence, was alive and active when this paper was written, 4½ years after the test (Table I).

PROCEDURES

The procedures carried out in the 10 patients started with the early-morning oral administration of 5.0 grams of potassium bicarbonate, which contains approximately 2.0 grams of potassium; during the next 3 hours, a study was made of its effect on the concentration of potassium and urea in the blood, and on serial electrocardiograms. The actual amount of potassium ingested by the 10 patients varied from 24 to 35 mgm. per kgm. of body weight. Breakfast was omitted and the patients remained in bed during the period of observation. Control samples of blood were withdrawn from a vein of the arm, and control electrocardiographic tracings were taken before the salt, dissolved in 300 ml. of water, was ingested. Subsequently, similar analyses of blood were made and electrocardiograms were taken at approximately 90 and 180 minutes. These were the procedures in 4 cases. In 1 of these, after the patient, H. S., had eaten his lunch, the blood studies and electrocardiograms were subsequently carried out at 8 hours (Tables I to III).

In 6 cases, in addition to the procedure described in the preceding paragraph, studies were made of the clearances of urea and potassium from the blood by the kidneys.

TABLE I

*Clinical data found on initial examination of patients**

Date of admission	Patient	Edema, grade 0–4	Blood pressure, range in 24 hours	Urine albumin, grade 0–4	Diagnosis
			mm. Hg		
10-30-41	M.S.	1	155–230 / 95–140	3–4	Chronic glomerulonephritis
11-28-41	P.K.	1–2	145–195 / 85–130	4	Subacute glomerulonephritis with nephrotic syndrome, necropsy
11-24-41	J.W.	1	110–140 / 60–90	4	Chronic glomerulonephritis with nephrotic syndrome
5-17-43	M.B.	0	150–200 / 110–130	2–3	Chronic glomerulonephritis, D.A.D. group 2
12-22-41	J.B.	2	130–190 / 70–110	3–4	Chronic glomerulonephritis with nephrotic syndrome, polycythemia vera
5-13-42	H.C.	0	110–180 / 70–110	2–3	Chronic glomerulonephritis
7-6-42	J.R.W.	0	80–105 / 40–65	2†	Chronic bilateral pyelonephritis
9-15-42	W.L.‡	0	180–215 / 100–135	3	Chronic glomerulonephritis, D.A.D. group 4
9-29-42	H.S.§	1	155–190 / 100–150	3	Bilateral congenital (?) hydronephrosis, necropsy ‖
4-15-43	L.G.	0	120–135 / 80–85	3–4¶	Multiple myeloma,¶ chronic diffuse nephritis

* Flocculation reactions for syphilis in serum of all 10 patients reported negative.
† Sediment contained pus, grade 3–4.
‡ Vascular retinitis, edema of disks.
§ Acute angiospastic retinitis.
‖ Necropsy elsewhere. Report kindly furnished by Dr. W. L. Ross, Jr., Yakima, Washington.
¶ Bence-Jones protein present in urine. Smear made from circulating blood has greasy quality and shows occasional myeloid immaturity. Serum protein 8.9 grams, serum albumin 4.0 grams, serum globulin 4.9 grams per 100 ml., respectively. Roentgenogram of skull reveals multiple punched-out regions of destruction.

The clearances in 2 periods of approximately 90 minutes' duration were determined in each of the 6 cases and correlated with the blood estimations and electrocardiographic tracings. Urea clearances were determined to give information as to the amount of renal tissue which was still functioning. The entire study in 4 cases was similar to that performed on normal controls and previously reported.

CLINICAL STUDIES

Vomiting occurred in only a single case 148 minutes after the patient, H. S., had ingested the salt. In this instance the vomitus was recovered; it contained only 0.2 gram of potassium. Three patients experienced mild epigastric discomfort or mild nausea soon after drinking the solution.

In previous studies, when large amounts of potassium salts were ingested by normal subjects, we (9), as well as a previous investigator (10), had noted that in certain cases paresthesias developed in the hands and feet while in other cases they did not develop. These paresthesias occurred when the concentration of potassium in the serum approximated 30 mgm. per 100 ml. It is interesting that in the present study, with a relatively small dose of 5.0 grams of potassium bicarbonate, paresthesias of the extremities developed in 4 patients, P. K., M. B., H. C. and H. S., when the content of potassium in the serum ranged from 31.1 to 35.8 mgm. per 100 ml. These findings suggested that the presence of paresthesias in the 4 patients was indicative of a decreased tolerance for potassium. Support for this viewpoint was the fact that paresthesias did not develop, nor did the serum potassium increase above 21.2 mgm. per 100 ml. in normal subjects who were given the same dose. In the remaining 6 cases paresthesias did not develop in the extremities; in 2, the maximal serum potassium varied from 19.9 to 27.1 mgm. per 100 ml. and in 4, it varied from 29.3 to 37.1 mgm. per 100 ml. Therefore, this symptom may not develop even when there is a decided increase of the concentration of serum potassium. Patient H. S. (Table II), the only patient to vomit during one of these experiments, was nervous throughout and was suffering from very severe uremia. The slow rise of the concentration of potassium in the serum and the later appearance of changes in the electrocardiogram lead one to surmise that absorption of potassium was at a considerably slower rate in that patient than in the remaining 9.

STUDIES OF THE BLOOD

We anticipated that the ingestion of 5.0 grams of potassium bicarbonate by our patients would produce changes in the concentration of potassium in the blood serum which would be different from those observed in normal subjects [1] (Figure 1) (8). In 9 of 10 patients the concentration of potassium in the serum, estimated before the salt was given, was abnormally increased to a range from 21.9 to

[1] Nineteen mgm. of potassium per 100 ml., a mean normal figure, = 4.9 m.eq. per 1.

TABLE II

Studies of urine and blood and clearance of blood urea and potassium of 10 patients

Date	Subject	Age, years, and sex	Weight	Height	Body surface	Experimental periods	Urine: Volume	Urine: Minute volume (V)	Urine: Potassium, Grams per 100 ml. (U)	Urine: Potassium, Total	Urine: Urea, Grams per 100 ml. (U)	Urine: Urea, Total	Blood: Whole blood, Hemoglobin, grams per 100 ml.	Blood: Whole blood, Urea, mgm. per 100 ml.	Blood: Whole blood, Creatinine, mgm. per 100 ml.	Clearances, ml. per minute: Serum clearance of potassium $\frac{U \times V}{S} \frac{1.73}{B.S.}$	Clearances, ml. per minute: Whole blood clearance of urea $\frac{U \times V}{B} \frac{1.73}{B.S.}$
			kgm.	*cm.*	*square meters*	*minutes*	*ml.*	*ml.*	*grams*	*grams*	*grams*	*grams*	*grams*	*mgm.*	*mgm.*	*ml.*	*ml.*
11-25-41	M.S.	20 F	56.6	163	1.60								13.1	150	5.6		
12-9-41	P.K.	17 M	82.3	181	2.02								11.7	148	6.4		
12-11-41	J.W.	17 M	55.9	176	1.69								10.8	74	1.3		
10-6-42	H.S.	20 M	74.5	181.6	1.95								8.1	340	19.2		
5-25-43	M.B.	38 M	68.9	170.2	1.80	99	270	2.7	0.191	0.516	0.508	1.372	13.5	90–90*	2.4	16	15
						89	180	2.0	0.258	0.464	0.627	1.129		90–84*		15	14
							450			0.980		2.501					
1-6-42	J.B.	58 M	66.0	174.5	1.80	105	80	0.76	0.344	0.275	1.240	0.992	14.2	147†	6.0	8	6
						87	110	1.26	0.375	0.413	1.130	1.243				13	9
							190			0.688		2.235					
5-22-42	H.C.	31 M	78.6	158	1.80	107	190	1.8	0.104	0.198	0.629	1.195	8.5	242†	14.4	6	6
						95	130	1.4	0.127	0.165	0.749	0.973		242‡		6	4
							320			0.363		2.168					
7-13-42	J.R.W.	18 M	55.7	160.6	1.58	110	155	1.4	0.084	0.130	0.617	0.956	9.1	280–280*	15.2	5	3
						88	210	2.4	0.073	0.153	0.574	1.205		280–282*		7	5
							365			0.283		2.161					
9-23-42	W.L.	32 M	70.0	185.4	1.94	96	148	1.5	0.077	0.114	0.905	1.339	7.4	188–194*	10.4	6	6
						83	110	1.3	0.104	0.114	0.947	1.042		194–192*		6	5
							258			0.228		2.381					
4-27-43	L.G.	40 M	75.7	171.5	1.89	98	176	1.8	0.151	0.266	0.635	1.118	8.1§	200–206*	12.0	8	5
						90	138	1.5	0.184	0.255	0.683	0.942		206–204*		7	5
							314			0.521		2.060					

* Concentration in whole blood at beginning and end of clearance period.
† Concentration in whole blood at beginning of clearance period.
‡ After 180 minutes after ingestion of $KHCO_3$.
§ Estimation of hemoglobin made after transfusion of 750 ml. of blood.

28.4 mgm. per 100 ml. (Figure 2). One and a half hours after the ingestion of the potassium bicarbonate the content of potassium in the serum of the 9 patients increased to a range from 23.1 to 37.1 mgm. per 100 ml. (Figure 2). The greatest increase, from 21.9 to 36.5, or 14.6 mgm., occurred in patient L. G. A similar increase of serum potassium was reported by Greene and his co-workers in a case of chronic nephritis with uremia. With this observation in L. G. and the distinct increase in patient M. S. from 26.7 to 37.1 mgm., we realized that even 5.0 grams of potassium bicarbonate might produce serious toxic effects, especially on the heart. It was rather surprising that the serum potassium in patient J. R. W., who had very serious renal disease, increased only to 27.1 mgm. In 3 hours (Figure 2) there was a decrease in the concentration of the potassium of the serum in 7 patients to values ranging from 24.0 to 35.4 mgm., but in 2 patients, J. B. and H. S., it increased 2.1 and 8.0 mgm., respectively. J. B. had taken a considerable amount of potassium nitrate as a diuretic previous to the tolerance test. The increase in patient H. S. from 23.1 mgm. at 1½ hours to 31.1 mgm. at 3 hours was marked. This late increase in the content of

TABLE III

Electrocardiographic findings

Patient	Date	Control period*						After 81 to 100 minutes†							After 175 to 186 minutes†						After 477 to 487 minutes†					
		Tracings, height of T waves, mm.‡ Leads§					Serum potassium, mgm. per 100 ml.	Tracings, height of T waves, mm.‡ Leads§					QRS segment	Serum potassium, mgm. per 100 ml.	Tracings, height of T waves, mm.‡ Leads§					Serum potassium, mgm. per 100 ml.	Tracings, height of T waves, mm.‡ Leads§					Serum potassium, mgm. per 100 ml.
		I	II	III	IV-R	CR-2		I	II	III	IV-R	CR-2			I	II	III	IV-R	CR-2		I	II	III	IV-R	CR-2	
M.S.	11-25-41	2+	2+	±	4+	7+	26.7	3+	4+	1+	6.5+	11+	‖	37.1	3+	3+	+	4+	4.5+	35.4						
P.K.	12-9-41	1+	3+	2+	4+	3+	26.6	1+	3+	2+	5+	5+		33.5	2+	4+	2+	5.5+	6+	33.2						
J.W.	12-11-41	2+	2+	±	3+	2+	22.2	3.5+	3+	1−	6+	3+		29.3	3.5+	3+	1−	8+	3+	25.6						
M.B.	5-25-43	1.8+	2.8+	1+	9+	7+	24.6	2+	3+	1.5+	6+	7.5+		35.8	1.8+	2.5+	1+	8+	5.5+	30.4	1.5+	2+	0.5+	7+	5+	24.4
J.B.¶	1-6-42	±	±	±	1.5+	6+	28.4	1−	+	1+	1+	7.5+		32.1	1−	+	1+	±	5+	34.2						
H.C.¶	5-22-42	3+	2+	1−	3.5+	6+	25.3	3.5+	2+	1.5−	7+	7.5+		31.1	3+	2+	1.5−	5+	6+	24.0	3+	2+	1−	4+	5+	
J.R.W.	7-13-42	2+	7+	5+	7.5+	5+	23.3	3.5+	8+	6+	9+	7+		27.1	2+	9+	6+	12+	7+	25.7	3+	8+	4+	7.5+	7+	21.7
W.L.¶	9-23-42	1−	1−	1+	1.5−	2+	16.8	1.5−	±	1+	2+	3+		19.9	2−	±	2+	4+	5.5+	16.9	1−	±	1+	±	4+	19.2
H.S.	10-6-42	±	2+	2+	4.5+	2+	22.2	0.5+	2.8+	2.9+	6+	3.2+		23.1	1+	3+	2.9+	11+	6+	31.1	0.5+	2+	2+	8+	3.5+	26.0
L.G.¶	4-27-43	2+	4+	2+	8+	5-6.5+	21.9	2+	4+	2.5+	7+	9-12+		36.5	1.5+	3+	2+	6.5+	7.5+	33.4	1+	3+	2+	5+	7.5-10+	24.9

* Electrocardiographic tracings, control.
† Electrocardiographic tracings at 81 to 100 minutes, at 175 to 186 minutes and at 477 to 487 minutes after ingestion of 5 grams of potassium bicarbonate.
‡ Ten millimeters is equal to 1 millivolt.
§ + = upright T waves; − = inverted T waves; ± = diphasic or iso-electric T waves.
‖ Intraventricular block and transient left axis deviation.
¶ Left axis deviation.

potassium in the serum of patient H. S. would appear to be due to a slow absorption of potassium from the intestinal tract. In 4 of the 9 patients, the concentration of potassium was estimated after 8 hours and it had decreased to 21.7 to 26.0 mgm., but in 3 of the 4 patients it was at a higher level than that observed in the original control serum. These results in 9 patients indicated a varied and unmistakably abnormal response to the ingestion of a relatively small amount of a potassium salt. The type of response was that of a decreased tolerance for potassium (Figures 1 and 2).

The findings, however, in patient W. L. were distinctly different from those in the 9 other patients. In fact, the alterations in the concentration of potassium in the serum (Figure 2) could not be distinguished from that obtained in a normal subject (Figure 1) after the ingestion of 5.0 grams of potassium bicarbonate. This response in a patient with such a severe degree of renal damage, the latter indicated by a blood urea clearance of 6 ml., surely informs us that the curve of serum potassium after the ingestion of a relatively small dose of a potassium salt may not reveal an altered potassium tolerance. The possibility of decreased absorption of the potassium salt from the intestine of this patient naturally must be considered. However, there is good evidence that intestinal absorption was satisfactory. This point will be discussed later.

STUDIES OF THE URINE AND BLOOD CLEARANCES OF UREA AND POTASSIUM

During the 2 clearance periods of approximately 1½ hours, 3 hours in all, after taking 5.0 grams of potassium bicarbonate by mouth, the mean volume of urine in the 6 patients whose urine was studied (Table II) was definitely less than that observed in normal subjects under the same experimental conditions. In fact the mean volume of urine excreted in 3 hours by 5 normal subjects was 497 ml. as compared with 316 ml. excreted by 6 patients. The concentrations of urea and potassium in the urine had a smaller range and were often lower than in the normal subjects. The mean of the total amounts of urea excreted was diminished. Also, the total amount of potassium eliminated was decreased and in patient W.L. was only 0.23 gram in 3 hours. If this amount of potassium was evenly excreted throughout the day it would total 1.8 grams in 24 hours, which approximates that excreted by a normal starving

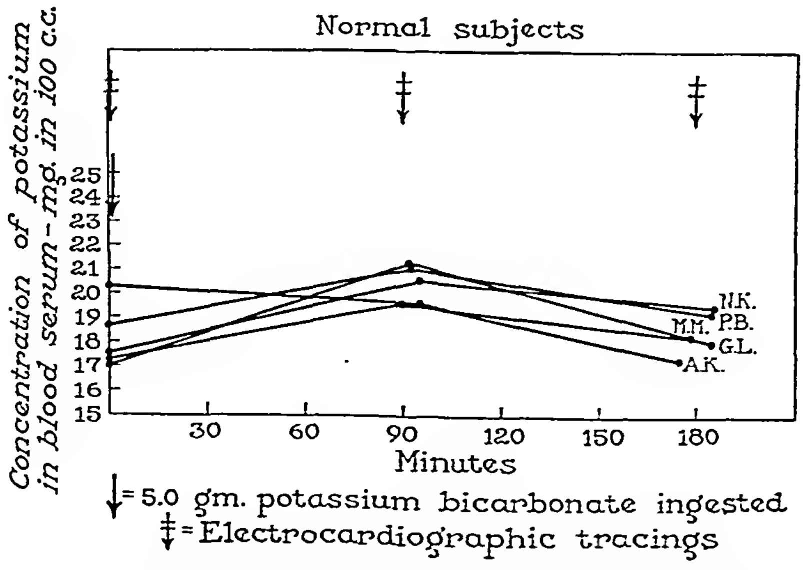

Fig. 1. Five Normal Subjects. The Concentration of Potassium in the Blood Serum Before and at 1½ and 3 Hours After the Ingestion of 5.0 Grams of Potassium Bicarbonate

Details of these studies reported in Proceedings of the Staff Meetings of the Mayo Clinic (8).

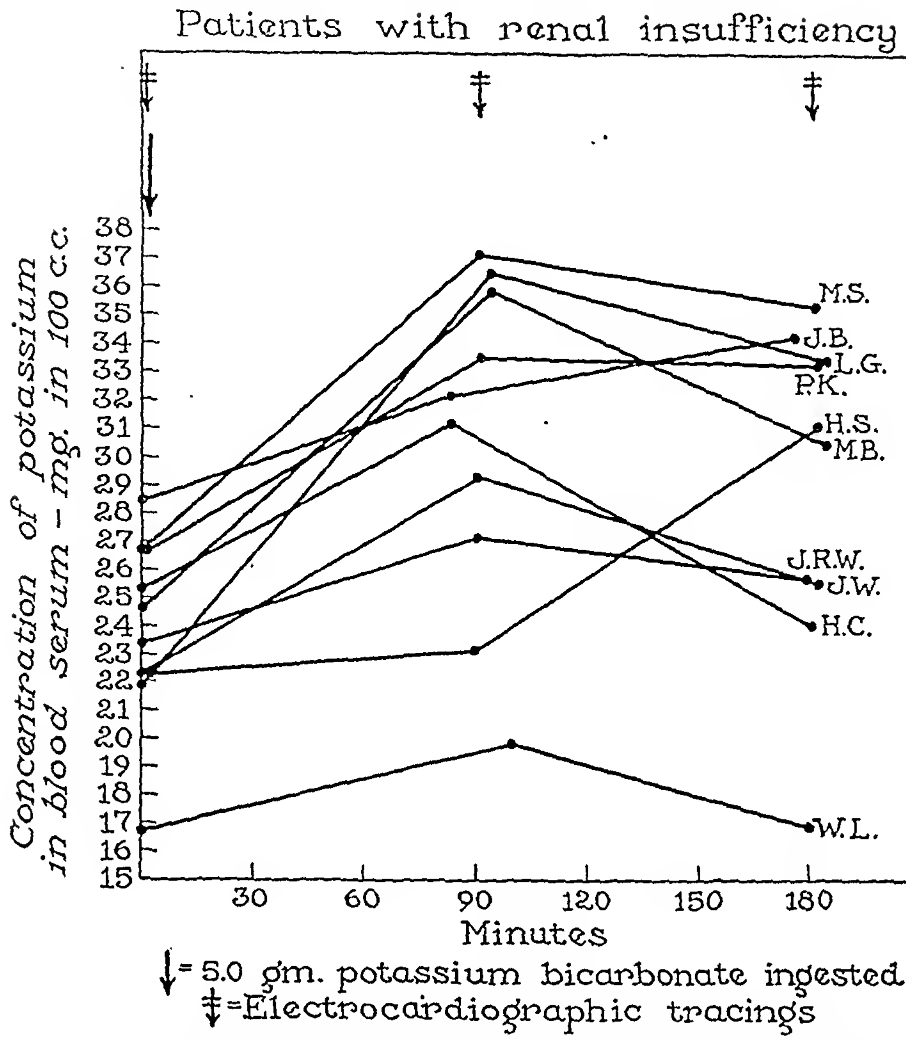

Fig. 2. Ten Patients with Renal Insufficiency. The Concentration of Potassium in the Blood Serum Before and at 1½ and 3 Hours After the Ingestion of 5.0 Grams of Potassium Bicarbonate

In 9 patients the serum potassium is distinctly increased over that observed in normal subjects. Compare with Figure 1. In 3 patients, M. S., M. B., and L. G., the serum potassium at 1½ hours increased to a potential toxic concentration. The curve of patient W. L. is similar to that of normal subjects.

subject.[2] Therefore, patient W. L. was unable in 3 hours to eliminate any of the extra potassium ingested. A similar calculation for 5 other patients revealed an excretion in 3 hours of 4 to 39 per cent of the extra potassium ingested. This is in distinct contrast to a range from 49 to 100 per cent in our studies on normal subjects.

The clearances of urea were greatly decreased to a range from 3 to 15 ml. (Table II). The clearances of potassium, ranging from 5 to 16 ml., however, were similar to those observed in normal subjects and in some patients who had renal insufficiency, when both subjects and patients were fasting and ingesting water only. It is clear, however, that the clearances of potassium, ranging from 5 to 16 ml., for our patients were distinctly less than those obtained, ranging from 29 to 67 ml., for normal subjects who had taken 5.0 grams of potassium bicarbonate. These facts indicate that in our patients there was a diminished and slower response of the kidneys to the stimulus of the potassium salt than in the normal subject. The kidneys of these patients were capable only of eliminating a dilute urine but were enabled by continuous excretion of potassium during the entire 24

[2] In Benedict's study (11), the starving subject on the first day of a prolonged fast excreted 1.63 grams of potassium in the urine.

hours to prevent its excessive retention in the blood.

The variability of the increases in the concentration of potassium in the serum of 6 patients contrasted markedly with the uniformity of the diminution of urinary excretion and clearances of potassium. The serum concentration, 1½ hours after ingesting the salt, varied from 19.9 to 36.5 mgm. or an increase of 5 to 92 per cent from a normal mean concentration of 19.0 mgm. in 100 ml. The mean reduction in the renal excretion of potassium in the 6 patients compared to that in normal subjects amounted to 1.09 grams or 32 per cent.

The decreased excretion and clearance of potassium in patient W. L. is unusually interesting, for, as stated in a previous section, the curve of the concentration of potassium in the blood serum (Figure 2) was identical with that observed in normal subjects (Figure 1) and one would therefore infer that this patient had a normal tolerance for potassium. However, the greatly reduced renal excretion of potassium (Table II) signified a decreased tolerance for potassium on the part of a single organ, the kidney. The results in patient W. L. also emphasized the important fact that there must be several organs that play a role in the internal regulation of potassium.

STUDIES OF THE ELECTROCARDIOGRAM [3]

There were 2 chief reasons for including serial electrocardiographic observations in the study of our 10 patients. The first was to ascertain whether 5.0 grams of potassium bicarbonate, when given to these patients, would cause alterations in the electrocardiogram similar to those observed in normal subjects. The second reason was to determine whether this relatively moderate dose of potassium bicarbonate would lead in patients to an excessive increase in the concentration of potassium in the serum and thus produce toxic effects on the heart. In each of the 10 patients serial electrocardiograms revealed some alterations in the T waves of the electrocardiogram (Table III). The alterations were often, though not invariably similar to those observed in normal subjects. Thus 5 normal subjects and 10 patients on whom this test was performed have revealed changes in the electrocardiogram which we think are due to a rapid increase of potassium in the myocardium. As was to be expected, changes observed in the patients were more varied than in the normal subjects; as a rule they persisted for a longer period and were sometimes observed for 8 hours after the taking of the salt. The changes due to potassium were sometimes more difficult to evaluate in patients than in the normal subject because a number of the control electrocardiograms of the patients were abnormal. For example, several patients had previously had severe hypertension and a tracing characteristic of left ventricular strain (Table III). Figures 3A and B show serial electrocardiograms with characteristic and varied alterations in 2 of our patients (J. R. W. and M. S.).

The electrocardiographic findings for patient H. S. (Table III) confirmed our previous clinical conception that potassium was more slowly absorbed from his intestine than in the 9 other patients. The curve of serum potassium slowly rose from 22.2 to 23.1 mgm. in 1½ hours, and to its peak, 31.1 mgm., in 3 hours and fell to 26.0 mgm. only after 8 hours. The concomitant changes in the electrocardiogram at 1½ hours revealed increased T waves, but at 3 hours these alterations in the T waves were much greater and appeared to be at their peak. Characteristic changes of the T waves, though less than at 3 hours, were present at 8 hours.

The electrocardiographic changes revealed in patient W. L. (Table III) at 1½ and 3 hours, though somewhat atypical, are of interest. It was previously pointed out that in this patient the concentration of potassium in the serum increased only 3.1 mgm. at 1½ hours, an increase similar to that found in normal subjects. Therefore we believe we have evidence in this patient that at least some of the potassium salt was rapidly absorbed from the intestine and produced concurrent effects in the blood serum and in the myocardium that are comparable with the response of the normal subject. The abnormally decreased renal excretion of potassium was the only evidence of a disturbed tolerance for this ion. The entire response in this patient could be explained by a decreased absorption of potassium from the intestinal tract. However, the evidence given earlier in this paragraph

[3] The authors are indebted to Dr. H. B. Burchell for this detailed interpretation of the electrocardiograms.

and the fact that some uremic patients have an abnormally low concentration of potassium in the serum and hence a low concentration in other tissues strongly support the viewpoint that normal absorption occurred and that the extra potassium entered tissues low in potassium content.

We have reliable evidence from the electrocardiographic and blood studies of patient M. S. (Figure 3B) that even the ingestion of 5.0 grams of potassium bicarbonate will produce toxic effects on the heart. In 1½ hours the content of potassium in the blood serum rose from 26.7 to 37.1 mgm. (Figure 2), an increase of 10.4 mgm., and the electrocardiographic tracings revealed early intraventricular block (Table III, Figure 3B). Previously we (12, 13), as well as other investiga-

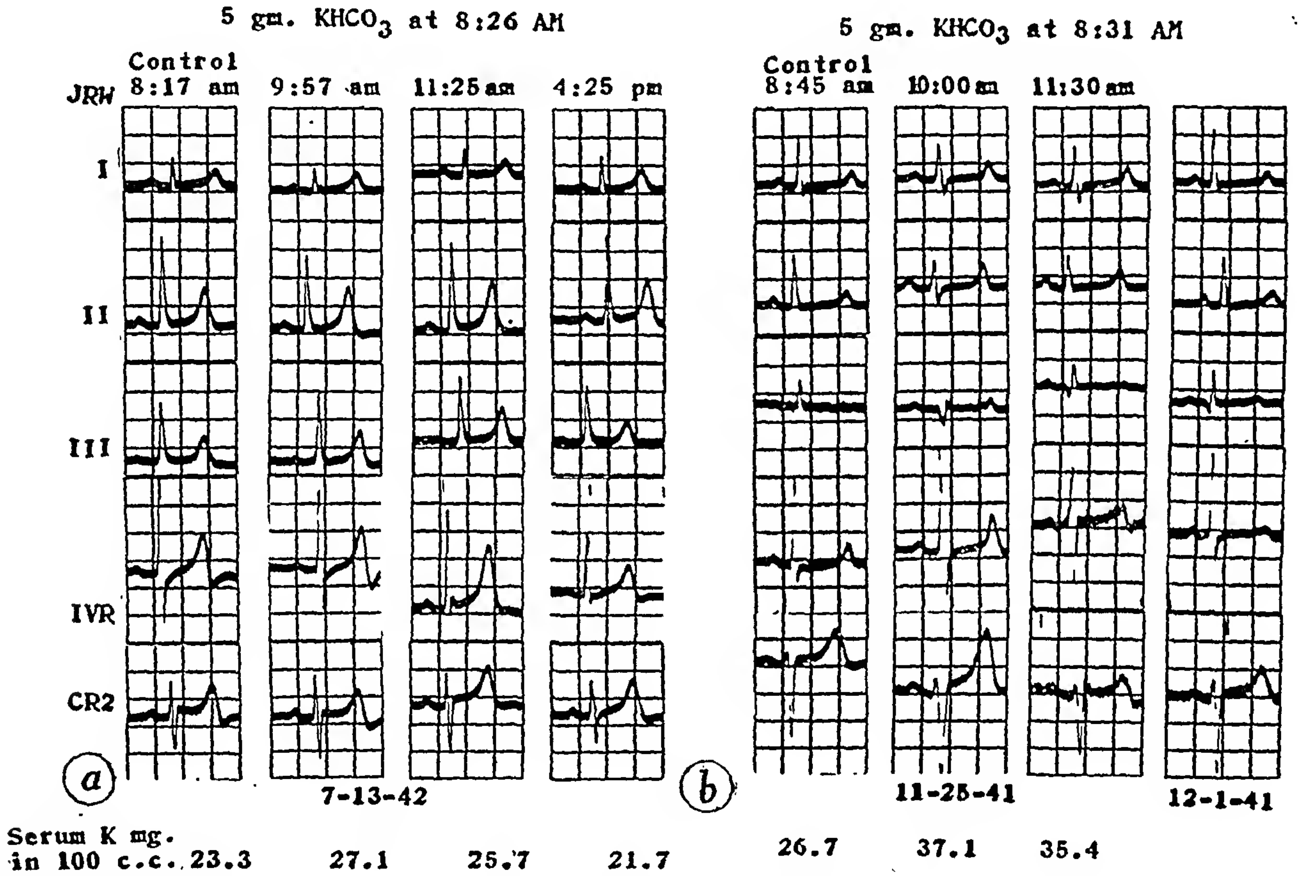

Fig. 3A. Patient J. R. W. Serial Electrocardiograms Showing the Effect of Increased Serum Potassium Levels

The patient did not reveal any abnormal cardiovascular findings. The first column, recorded shortly before administration of potassium, is normal. Following the ingestion of the potassium salt, the records show increases of the voltage of the T wave (2 mm. in lead II, 4 mm. in lead IV–R). It is to be noted that while the potassium level has fallen 1.4 mgm. between 9:57 and 11:25 a.m., the T waves are at their highest at the latter time. In IV–R there are loss of the late negativity and a slight elevation of the R–T interval. The QRS complexes and the various time intervals do not show any significant change.

B. Patient M. S. Serial Electrocardiograms Showing the Effect of Increased Levels of Potassium in the Serum

The control tracing is an essentially normal electrocardiogram. With the increase of serum potassium level there is a concomitant increase in the height of the T wave and in the length of the QRS interval (from 0.07 to 0.09 second). The QRS has become more biphasic and suggests the beginning of the intraventricular conduction defect which is so characteristic of the tracing in patients having potassium concentrations approaching the lethal level. Such a tracing has not been observed previously with such a small dose of a potassium salt. Attention is drawn to the characteristic sharply peaked, narrow-base configuration of the T waves as seen in leads II and IV–R of the second column. The Q–T intervals are respectively 0.48, 0.48, 0.44, and 0.44 second and the corresponding R–R times are 1.05, 0.90, 1.10, and 1.15 seconds respectively. On November 26 the serum calcium was 8.4 mgm. per 100 ml.

tors (14), have reported a similar degree of hyperpotassemia and electrocardiographic findings of intraventricular block among patients suffering from severe uremia and not receiving therapeutic doses of potassium salts. These patients died shortly afterward because of the toxic effect of the potassium on the heart. Fortunately, in this patient at the end of 3 hours the evidence of intraventricular block had almost disappeared although the concentration of potassium in the serum was still high, 35.4 mgm. Several days later, electrocardiograms were taken and proved to be identical with those observed before the potassium salt was ingested. Sharpey-Schafer in 1943 (7) reported the temporary induction of intraventricular block in a patient who had myocardial degeneration and had ingested a much larger dose, 15 grams of potassium salts. Our experience with patient M. S. affords objective evidence that in chronic nephritis there may be a marked decrease of the tolerance for potassium and hence the administration of potassium salts may be a dangerous procedure. On the other hand, it is significant that in the remaining 9 patients, some of whom had very severe renal insufficiency, the electrocardiogram did not reveal definite intraventricular dysfunction.

COMMENT

In 4 cases paresthesias developed in the extremities, a fact which we think is clinical evidence of a lowered potassium tolerance, because these symptoms were observed previously only in subjects to whom much larger doses of potassium had been administered. In all of our 10 patients the excretion of potassium by the kidney was decreased, and in 9 there was an abnormal rise of the curve of serum potassium. Both results indicated a definite decrease of tolerance.

The concentration of potassium in the serum rose to potentially dangerous levels in 3 patients, but only in a single instance did it produce a demonstrable toxic effect on the cardiac muscle. In the 3 patients, the extra potassium ingested was presumably distributed chiefly in the extracellular fluid of the body. Calculation of the distribution of potassium in 1 of these, patient L. G., was as follows: His body weight amounted to 75.7 kgm. If 20 per cent of the body weight may be considered to represent the volume of extracellular fluid, in this subject it would amount to, therefore, 15,000 ml. If then 2.0 grams of potassium, less that excreted in the urine, is dissolved in 15,000 ml. of fluid, the estimated increase in the concentration of potassium would be 12 mgm. per 100 ml.[4] The observed increase in the patient's serum was 14.6 mgm. per 100 ml., which is in close agreement with the calculated value.

In 7 patients the potassium ingested was presumably distributed, in varying proportions, between the extracellular and the intracellular fluid. However, in a single patient a large proportion apparently entered rapidly into the intracellular fluid. This result would lead one to believe that the stores of potassium in the cells of this patient were depleted, despite the presence of severe renal insufficiency. Our results, therefore, indicate a marked variation in the distribution of potassium between the 2 reservoirs of body fluid.

The small increase in the serum curve of patient W. L. also suggests the possibility that in certain patients who have uremia there may be an actual increase of tolerance for potassium. In this connection several observers have recorded concentrations of serum potassium distinctly below the normal range in patients who have renal disease (12). We reported a content of 12.8 mgm. per 100 ml. of serum in 1 such patient (15) and Brown, Currens and Marchand (16) reported a concentration of 7.8 mgm. in the case they reported.

In the present study we have demonstrated the occurrence of a decreased tolerance for potassium. This is in agreement with the findings in the blood serum in 2 cases of renal insufficiency published by Greene and his co-workers. Considerable evidence also has been advanced for the presence of both a normal and an increased tolerance. Further research is necessary to prove the occurrence of these latter conditions. Studies of potassium tolerance are also needed in the states of inanition or semistarvation, which develop rather frequently among patients suffering from chronic uremia.

In a previous paper (17) we recommended caution in administering potassium salts to a nephritic patient who has edema and in whom the concentration of urea in the blood is 100 mgm. or

[4] $\frac{2{,}000 \text{ mgm.} - 266 \text{ mgm.}}{15{,}000} = 12 \text{ mgm. per } 100 \text{ ml.}$

more per 100 ml. In 9 cases, in our present series, in which the tolerance for potassium was reduced, the urea content of the blood varied from 90 to 340 mgm. per 100 ml. We still rely on this chemical observation as a precautionary measure. Therefore, it is suggested that a potassium tolerance test be carried out if a patient has a blood urea of 100 mgm. per 100 ml. and the results should provide evidence as to whether or not a potassium salt could be safely administered. Winkler, Hoff, and Smith (5) have emphasized the point that oral administration of potassium salts to patients who have nephritic edema is a reasonably safe procedure and our results support their contention, especially when there is an adequate renal excretion of the metabolites, urea, sulfate, and creatinine. Fortunately the majority of patients who have nephritic edema will reveal a concentration of urea in the blood which is much less than 100 mgm. per 100 ml.

SUMMARY

A diminished tolerance for potassium has been demonstrated in a series of uremic patients. Other results of this study suggest the presence of a normal tolerance and even an increased tolerance for potassium in certain phases of uremia but further research is necessary to prove or disprove their actual occurrence.

BIBLIOGRAPHY

1. Zwemer, R. L., and Truszkowski, Richard, Factors affecting human potassium tolerance. Proc. Soc. Exper. Biol. & Med., 1936, 35, 424.
2. Wilder, R. M., Kendall, E. C., Snell, A. N., Kepler, E. J., Rynearson, E. H., and Adams, Mildred, Intake of potassium, an important consideration in Addison's disease: a metabolic study. Arch. Int. Med., 1937, 59, 367.
3. Jaffe, I., and Byron, C., Potassium tolerance test as aid in diagnosis of adrenal insufficiency. J. Clin. Endocrinol., 1942, 2, 339.
4. Greene, J. A., Levine, Harry, and Johnston, G. W., Is the potassium tolerance curve of value in the diagnosis of adrenal cortical insufficiency in man? Endocrinology, 1940, 27, 375.
5. Winkler, A. W., Hoff, H. E., and Smith, P. K., The toxicity of orally administered potassium salts in renal insufficiency. J. Clin. Invest., 1941, 20, 119.
6. Thomson, W. A. R., The effect of potassium on the heart in man. Brit. Heart J., 1939, 1, 269.
7. Sharpey-Schafer, E. P., Potassium effects on T wave inversion in myocardial infarction and preponderance of a ventricle. Brit. Heart J., 1943, 5, 80.
8. Keith, N. M., and Osterberg, A. E., The human tolerance for potassium. Proc. Staff Meet., Mayo Clin., 21: 385-392 (Oct. 2) 1946.
9. Keith, N. M., Osterberg, A. E., and Burchell, H. B., Some effects of potassium salts in man. Ann. Int. Med., 1942, 16, 879.
10. Arden, Felix, Experimental observations upon thirst and on potassium overdosage. Australian J. Exper. Biol. & M. Sc., 1934, 12, 121.
11. Benedict, F. G., A study of prolonged fasting. Washington, D. C., Carnegie Inst., 1915, p. 203.
12. Keith, N. M., King, H. E., and Osterberg, A. E., Serum concentration and renal clearance of potassium in severe renal insufficiency in man. Arch. Int. Med., 1943, 71, 675.
13. Keith, N. M., Burchell, H. B., and Baggenstoss, A. H., Electrocardiographic changes in uremia associated with a high concentration of serum potassium; report of three cases. Am. Heart J., 1944, 27, 817.
14. Marchand, J. F., and Finch, C. A., Fatal spontaneous potassium intoxication in patients with uremia. Arch. Int. Med., 1944, 73, 384.
15. Rosenberg, E. F., Keith, N. M., and Wagener, H. P., Diffuse arterial disease with hypertension; two unusual cases of contrasting types. Arch. Int. Med., 1938, 62, 461.
16. Brown, M. R., Currens, J. H., and Marchand, J. F., Muscular paralysis and electrocardiographic abnormalities resulting from potassium loss in chronic nephritis. J. A. M. A., 1944, 124, 545.
17. Keith, N. M., and Binger, M. W., Diuretic action of potassium salts. J. A. M. A., 1935, 105, 1584.

METHODS OF CHEMICAL ANALYSIS EMPLOYED IN THE PRESENT STUDY

Whole blood

Hemoglobin—Sanford, A. H., Sheard, Charles, and Osterberg, A. E., The photelometer and its use in the clinical laboratory. Am. J. Clin. Path., 1933, 3, 405.

Urea—Van Slyke, D. D., and Cullen, G. E., A permanent preparation of urease, and its use in the determination of urea. J. Biol. Chem., 1914, 19, 211.

Creatinine—Folin, Otto, Laboratory manual of biologic chemistry with supplement. Ed. 3, New York, D. Appleton & Company, 1922, pp. 149, 245 and 247.

Serum

Potassium—Kramer, Benjamin, and Tisdall, F. F., A clinical method for the quantitative determination of potassium in small amounts of serum. J. Biol. Chem., 1921, 46, 339. This method in our hands has checked with gravimetric procedures. The precipitation by sodium cobaltinitrite was allowed to proceed for 45 minutes at icebox temperature in order to obtain a good granular precipitate. If this and other steps are rigorously adhered to, the method is satisfactory.

Calcium—Clark, E. P., and Collip, J. B., A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. J. Biol. Chem., 1925, 63, 461.

Total proteins—Macro-Kjeldahl technic of estimation of total nitrogen and total protein, calculated after subtracting nonprotein nitrogen as estimated by the micro-Kjeldahl method.

Albumin and globulin fractions—Howe, P. E., The determination of proteins in blood; a micro method. J. Biol. Chem., 1921, 49, 109. Kingsley, G. R., A rapid method for the separation of serum albumin and globulin. J. Biol. Chem., 1940, 133, 731.

Urine

Urea—Same method as for whole blood.

Potassium—Same method as for estimation in serum except that urine is ashed before precipitation.

THE EFFECTS OF VAGOTOMY ON THE PHYSIOLOGY OF THE STOMACH IN PATIENTS WITH PEPTIC ULCER [1]

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Since January of 1943 bilateral section of the vagus nerves to the stomach has been performed in this clinic on 160 patients with benign duodenal, jejunal, and gastric ulcers. The technic employed to obtain complete bilateral vagus section and the clinical results have been described elsewhere (1 to 4). During the course of this work there arose the opportunity to study the effect of vagotomy on the continuous night secretion of gastric juice in the empty stomach, on the gastric secretory response to histamine, caffeine, insulin, and sham feeding, and on gastric motility in ulcer patients. A report dealing with a portion of these studies has appeared recently (5). The present paper presents the results of night secretion tests on 37 patients with diseases other than benign ulcers and on 42 patients who underwent vagotomy for benign ulcer. It describes the gastric secretory response to insulin hypoglycemia and sham feeding before and after vagotomy in 23 ulcer patients and the effect of vagotomy on motility in 13 such patients.

In general, the patients who underwent vagotomy and are presented here had ulcer symptoms of many years' duration. With only a few exceptions they had all experienced typical ulcer pain and in most a crater had been demonstrated by gastroduodenal x-ray examination. A majority had suffered pyloric obstruction, hemorrhage, or perforation. They were either refractory to medical management or had been proved unable to follow such a regime. Thus, in every case, the ulcer represented a serious affliction. The patients were led to accept vagus section because of refractory pain, recurrent hemorrhage, pyloric obstruction, or the prospect of cure of their ulcer disease without the necessity for long-term medical management.

Bilateral section of the vagus nerves to the stomach was performed at or just above the level of the diaphragm. In those cases which required gastroenterostomy because of appreciable pyloric obstruction, the vagus section was done through the abdominal incision. In one case in this series (J. A.) a transabdominal subtotal gastrectomy and vagus section were done.

The results of this procedure have so far been extremely gratifying. In many cases x-ray examination has demonstrated progressive healing of large ulcers over a period of 3 to 12 weeks following operation. Prompt relief of ulcer pain following vagotomy is characteristic. All but 4 of the 42 patients reported here experienced immediate relief of all pain without recurrence. Of these 4, 2 (M. H. and J. W.) have had abdominal pain of an atypical character after vagotomy, and it is probable that this pain is not on the basis of ulcer disease. The third patient (H. K.) has had a single brief bout of epigastric distress since operation. A fourth patient continued to have symptoms after operation, and when a total gastrectomy was done elsewhere 8 months later these were determined to be due to obstruction rather than to persistent ulceration (6).

Fifteen of these 42 patients had postoperative diarrhea of varying degree, ranging from 4 to 5 loose stools per day to replacement of preoperative constipation by normal bowel habits. Actual diarrhea has not persisted longer than 3 to 6 weeks following vagotomy. In some patients the relief of preoperative habitual constipation has constituted a pleasant surprise and has persisted for months and to the present time. Postoperative pain in the thoracotomy wound has, with few exceptions, disappeared within 3 weeks following operation.

METHODS

1. *The continuous 12-hour night secretion of gastric juice*

At 9:00 p.m. the stomach was emptied with a Levine tube, or with an Ewald tube if its larger diameter was necessary. The stomach was lavaged with water until the return was clear. A Levine tube was placed in the

[1] This work has been aided by grants from Mr. Andrew E. Wigeland and from the Douglas Smith Foundation for Medical Research of The University of Chicago.

stomach and was left in place under continuous Wangensteen suction for the subsequent 12 hours. Most of these patients were accustomed to intubation and the inlying Levine tube did not upset them nor interfere with their sleep. Approximately 20 per cent, those who could not rest soundly without medication, received 0.12 gram of sodium luminal at 9:00 p.m. subcutaneously. While the tube was in place the patients were not exposed to the sight, smell, or taste of food. The juice aspirated from the stomach during the night was collected at 9:00 a.m. and any specimens which contained particles of food were discarded. The volume of each specimen was measured and the free and total acid were determined in terms of "clinical units" by titration with 0.1 N NaOH to the change of color with Topfer's reagent and phenolphthalein, respectively. The figures on night secretion in patients with diseases other than benign ulcer (Tables I to III) represent the results derived from an aspiration conducted on each patient on the night before operation. All figures on the night secretion in ulcer patients before and after operation (Tables IV to VI) represent in each case the average of 3 or more night secretion tests performed before and again within 2 weeks after operation.

TABLE I

The continuous night secretion of gastric juice in patients with carcinoma of the stomach

Patient no.	Age	Volume	Free acidity, clinical units	Total acidity, clinical units	Total acid output	Volume parietal cell output
		ml.			*mM.*	*ml.*
1	56	260	0	40	10	60
2	31	220	21	39	9	54
3	74	84	0	44	4	24
4	74	75	0	18	1	6
5	54	290	8	22	6	36
6	60	500	0	25	13	78
7	60	200	11	30	6	36
8	60	290	0	38	11	66
9	30	430	0	41	18	108
10	56	444	0	43	19	114
11	68	800	25	55	44	27
12	64	400	17	30	12	72
Average		333	7	35	13	57

TABLE II

The continuous night secretion of gastric juice in patients with cholelithiasis and chronic cholecystitis

Patient no.	Age	Volume	Free acidity, clinical units	Total acidity, clinical units	Total acid output	Volume parietal cell output
		ml.			*mM.*	*ml.*
1	27	630	13	31	20	120
2	49	100	0	24	2	12
3	27	360	12	26	9	54
4	51	175	13	30	5	30
5	45	445	0	27	12	72
6	61	600	0	17	10	60
7	59	140	48	69	10	60
8	39	300	10	25	8	48
9	49	260	24	53	14	64
10	62	225	42	81	18	108
11	46	615	29	48	30	180
12	42	620	7	31	19	114
13	53	200	32	55	11	66
14	62	360	0	12	4	24
Average		359	16	38	12	72

TABLE III

The continuous night secretion of gastric juice in patients without gastric or duodenal ulcer

Patient no.	Age	Diagnosis	Volume	Free acidity, clinical units	Total acidity, clinical units	Total acid output	Volume parietal cell output
			ml.			*mM.*	*ml.*
1	53	Common bile duct stenosis	310	8	15	5	30
2	58	Ca. of rectum	700	62	78	55	330
3	57	Polyp of colon	480	31	48	23	139
4	51	Hernia	330	13	27	9	54
5	51	Functional bowel distress	378	44	69	26	157
6	41	Retroperitoneal sarcoma	442	0	57	25	151
7	45	Ca. of breast	400	35	65	26	157
8	37	Endometriosis	420	24	35	15	90
9	16	Terminal ileitis	810	42	56	45	271
10	55	Adhesions of colon	640	37	59	38	229
11	34	Polyp of colon	336	20	30	10	60
Average			477	29	49	25	152

It seemed valuable to express the night secretion of the stomach in terms of "Total acid output in millimoles" and "volume of parietal cell output in ml." Since the total acid concentration of the gastric juice, expressed as "clinical units," is numerically equal to the number of millimoles of HCl per liter in the gastric juice, the following is true.

(Total acid in C.U.) × (Vol. of night secretion in liters) = (Total HCl output in millimoles)

Moreover, it is known that pure parietal cell secretion contains very close to 166 mM. per liter of HCl (7). It may therefore be said that:

$$\frac{(\text{Total HCl output in mM.})}{166} \times 1{,}000 = (\text{Total HCl output in mM.}) \times 6.02 = (\text{Volume of parietal cell output in ml.}).$$

2. *The insulin test*

A Levine tube was inserted in the fasting stomach before breakfast and the stomach emptied. The patient lay quietly in bed in a comfortable semirecumbent position. He was shielded from outside stimuli, especially from food. The gastric juice was collected continuously for

a period of approximately 2 hours. The amount aspirated in each 10-minute period was measured as to volume and free and total acidity. At the end of 30 minutes the patient received 20 or 30 units of crystalline insulin subcutaneously. Blood sugar levels were determined at ½ hour and 2 hours after the beginning of the test. A majority of patients showed a mild insulin reaction about 1 to 1½ hours after receiving the injection. One or 2 insulin tests were done on each patient before and again within 2 weeks after vagotomy.

TABLE IV

The continuous 12-hour night secretion of gastric juice before and after transthoracic section of the vagus nerves to the stomach for duodenal ulcer

Patient	Preoperative night secretion					Postoperative night secretion				
	Volume	Free acid conc.	Total acid conc.	Total acid output	Volume parietal cell output	Volume	Free acid conc.	Total acid conc.	Total acid output	Volume parietal cell output
	ml.	*C.U.*	*C.U.*	*mM.*	*ml.*	*ml.*	*C.U.*	*C.U.*	*mM.*	*ml.*
1. T. M.	1,138	78	81	92	553	494	25	37	18	108
2. J. S.	1,373	24	47	65	373	1,065	36	55	59	355
3. F. V.	1,029	61	79	81	488	670	43	61	41	246
4. J. D.	612	42	54	33	199	548	7	68	37	224
5. E. K.	1,193	15	58	69	416	1,180	42	81	95	575
6. J. M.	927	53	68	63	378	323	9	30	10	60
7. M. H.	578	25	48	28	168	312	26	57	18	108
8. H. K.	723	39	58	42	252	65	24	48	3	18
9. H. M.	775	36	54	42	252	428	6	44	19	114
10. N. K.	717	55	81	58	349	400	19	51	20	120
11. E. T.	786	44	61	48	288	358	0	30	11	66
12. R. V.	1,043	82	98	102	612	486	61	82	40	241
13. W. W.	756	54	72	55	331	523	15	56	29	175
14. C. O.	642	66	81	52	313	212	2	66	14	84
15. W. K.	1,086	46	63	68	407	295	11	25	7	42
16. G. K.	460	31	54	25	149	248	14	42	10	60
Average	865	47	66	58	346	475	21	52	27	162

TABLE V

The continuous 12-hour night secretion of gastric juice before and after transabdominal vagotomy plus posterior gastroenterostomy for duodenal ulcer

Patient	Preoperative night secretion					Postoperative night secretion				
	Volume	Free acid conc.	Total acid conc.	Total acid output	Volume parietal cell output	Volume	Free acid conc.	Total acid conc.	Total acid output	Volume parietal cell output
	ml.	*C.U.*	*C.U.*	*mM.*	*ml.*	*ml.*	*C.U.*	*C.U.*	*mM.*	*ml.*
1. G. H.	1,838	19	42	77	464	630	0	17	11	66
2. J. G.	881	43	75	66	397	663	57	83	55	331
3. T. B.	1,305	33	32	42	253	617	0	37	23	138
4. N. W.	623	56	56	35	210	370	33	55	20	120
5. L. N.	998	18	44	44	264	880	50	73	64	384
6. A. F.	545	14	39	21	126	602	21	32	19	116
7. J. O.	856	62	84	72	432	540	2	26	14	84
8. T. B.	1,160	21	39	45	271	940	2	35	33	198
9. F. H.	1,095	48	67	74	445	770	0	39	30	180
10. I. C.	1,642	35	48	79	475	910	0	16	15	90
11. F. L.	755	60	65	49	295	500	31	40	20	120
12. S. N.	768	28	38	29	175	390	0	36	14	72
13. E. L.	1,053	47	65	69	415	1,146	34	57	65	391
14. H. F.	1,142	44	55	63	379	395	35	44	18	108
15. H. W.	1,241	32	51	62	373	902	0	29	26	157
16. W. S.	1,165	35	52	61	367	893	25	48	43	259
17. J. P.	405	23	45	18	108	517	14	52	27	163
18. J. A.	627	35	66	41	247	347	0	57	20	120
Average	1,000	36	54	53	317	665	17	43	29	172

TABLE VI

The continuous 12-hour night secretion of gastric juice before and after transthoracic section of the vagus nerves to the stomach for jejunal ulcer

Patient	Preoperative night secretion					Postoperative night secretion				
	Volume	Free acid conc.	Total acid conc.	Total acid output	Volume parietal cell output	Volume	Free acid conc.	Total acid conc.	Total acid output	Volume parietal cell output
	ml.	*C.U.*	*C.U.*	*mM.*	*ml.*	*ml.*	*C.U.*	*C.U.*	*mM.*	*ml.*
1. J. V.	1,020	30	43	44	265	—	—	—	—	—
2. A. W.	897	41	54	48	249	286	0	25	7	42
3. M. B.	1,021	22	45	46	277	412	14	36	15	90
4. C. B.	200	13	36	7	42	177	0	30	5	30
5. A. W.	566	0	21	12	62	361	0	23	8	42
6. G. P.	1,126	12	36	41	247	233	0	19	4	24
7. M. S.	310	6	25	8	48	395	0	30	12	72
8. J. W.	1,220	20	40	48	289	338	0	19	6	36
Average	795	18	38	32	185	315	2	26	8	42

3. *The sham meal test*

Levine tube was placed in the fasting stomach before breakfast and the stomach emptied. The patients sat up comfortably in bed and his surroundings were made as agreeable as possible. The gastric secretion was collected over three 10-minute periods as a control. Then the patient was presented with an attractively prepared, appetizing "breakfast in bed," consisting of a small glass of orange juice, 2 slices of bacon, 2 pieces of lightly buttered toast, and 1 cup of coffee with cream and sugar. The patient proceeded just as he would with any ordinary breakfast, except that after tasting and chewing it he expectorated the food into a tall container, so chosen as to prevent him from seeing the masticated food it held. The gastric secretion was collected continuously and portioned into 10-minute samples for 1 to 1½ hours after the test breakfast was begun. The volume and free and total acidity of each sample were determined. If the patient swallowed any of the test breakfast, the test was discarded. The patient usually spent 10 to 15 minutes from start to finish of the test meal. One or 2 sham meal tests were done on each patient before and after vagotomy.

4. *Gastric motility*

A Miller-Abbott tube was inserted into the fasting stomach and the balloon was inflated to a pressure of 15 to 25 cm. of water. A record of gastric motility was obtained on a smoked drum by means of a water manometer connected to this balloon. During each test the patient lay supine or on his right or left side. No medication was given to these patients during these tests, but most of them slept at least part of the time. At each test it was determined whether the patient had normal "tonus" waves and normal "hunger contraction." It was also determined whether the patient could feel and identify distention of the balloon beyond the amount used for recording and whether the passage of the contraction waves were accompanied by any sensation. The motility test was performed once before and again once within 2 weeks after vagus section.

RESULTS

1. *The continuous 12-hour night secretion of gastric juice*

It will be seen from perusal of Tables I to IV that there is a marked difference between the ulcer patients and those without benign ulcers in both quantity and acidity of juice aspirated by continuous suction during the 12-hour nocturnal period. The average volume in 12 patients with carcinoma of the stomach was 333 ml. with a free acidity of 7 and a total of 35 clinical units. That of 14 patients with cholelithiasis was 359 ml. with a free and total acidity of 16 and 38 units. Eleven patients with miscellaneous other diseases averaged a night secretion of 477 ml. with a free acidity of 29 and a total of 49 clinical units. These figures contrast with those obtained in 42 patients with benign ulcers. The average night secretion of the 16 patients presented in Table IV, all of whom had little or no element of pyloric obstruction, was 865 ml. with a free acid of 47 and a total of 66 clinical units. In Table V, consisting of patients with moderate to marked pyloric obstruction, the average volume of nocturnal secretion is 1,000 ml. free acidity 36 and total 53 clinical units. Even some of the patients with jejunal ulcers following gastric resection (J. V. and G. P. in Table VI) show an elevated acid output. As determined by this method the difference in acid output between pa-

tients with benign ulcers and without is great. The exceptions to the rule are few.

Analysis of Tables IV to VI also reveals that section of the vagus nerves to the stomach, with few exceptions, causes a marked reduction in the volume and acidity of the nocturnal gastric aspiration. The average total acid output of the stomach in 16 patients (Table IV) was halved by vagotomy. Two of these patients, however (J. D. and E. K.), showed no reduction. It is not surprising that the average acidity of the aspirate was reduced after vagotomy plus gastroenterostomy (Table V), but it should be noted that the volume of aspirate was also reduced, despite regurgitation of duodenal juices through the gastroenterostomy stoma. The night secretion of all but 1 of 7 patients with jejunal ulcers (Table VI) was also reduced after vagotomy.

TABLE VII

Gastric secretory response of duodenal ulcer patient (F.V.) to insulin-induced hypoglycemia before and after transthoracic section of the vagus nerves to the stomach

Time	Volume	Free acid	Total acid	Remarks
	ml.	*C.U.*	*C.U.*	
Four days before operation				
8:30–8:40 a.m.	31	69	88	
8:50	23	48	69	
9:00	24	51	70	9:00 20 units insulin given subcutaneously
9:10	31	43	62	
9:20	13	31	47	
9:30	11	24	43	
9:40	9	15	34	
9:50	19	8	27	
10:00	8	9	28	
10:10	38	40	62	
10:20	40	81	105	
10:30	33	107	130	
10:40	39	105	127	
10:50	41	111	129	
11:00	33	112	131	
Eight days after operation				
8:30–8:40 a.m.	45	44	56	
8:50	40	51	59	
9:00	16	50	60	9:00 20 units insulin given subcutaneously
9:10	7	53	65	
9:20	8	54	65	
9:30	7	45	62	
9:40	2	21	36	
9:50	15	26	40	
10:00	2	31	37	
10:10	—	—	—	
10:20	6	38	49	
10:30	2	39	53	
10:40	5	26	40	
10:50	5	20	37	

TABLE VIII

Gastric secretory response of duodenal ulcer patient (J. O.) to insulin-induced hypoglycemia before and after transabdominal vagotomy plus posterior gastroenterostomy

Time	Volume	Free acid	Total acid	Remarks
	ml.	*C.U.*	*C.U.*	
Three days before operation				
8:00–8:10 a.m.	58	90	101	
8:20	28	86	97	8:30 Fasting blood sugar 52 mgm. per cent
8:30	56	75	95	
8:40	27	85	95	8:30 30 units insulin given subcutaneously
8:50	40	83	97	
9:00	36	65	80	
9:10	26	54	70	
9:20	4	70	85	
9:30	36	93	112	9:30 Feels hot and hands and feet are numb. Flushed. Slight tremor
9:40	40	95	113	
9:50	42	113	132	
10:00	42	124	136	10:00 Blood sugar 20 mgm. per cent
10:10	25	124	136	
10:20	25	113	129	
10:30	25	110	129	
Twelve days after operation				
8:00–8:10 a.m.	3	6	17	
8:20	12	0	13	8:30 Fasting blood sugar 76 mgm. per cent
8:30	10	0	11	
8:40	10	0	12	8:30 30 units insulin given subcutaneously
8:50	12	0	17	
9:00	18	0	9	
9:10	17	0	12	
9:20	17	0	11	
9:30	8	0	12	
9:40	15	0	20	
9:50	8	0	10	
10:00	8	0	16	
10:10	10	0	15	10:00 Blood sugar 44 mgm. per cent. Feels weak
10:20	9	0	15	
10:30	8	0	12	

2. *The insulin test*

The protocols of representative insulin tests are presented in Tables VII to IX. There was no consistent response to insulin hypoglycemia in terms of volume of 10-minute secretions. There was, however, a regular response in terms of acidity of the aspirate following the injection of insulin. Beginning 60 to 90 minutes following injection, there was usually a marked rise in free and total acidity of the aspirated juice. In reviewing this series of tests it appeared that there was a random variation of the acidities of the samples that amounted to approximately 20 clinical units in

TABLE IX

Gastric secretory response of duodenal ulcer patient (G. H.) to insulin-induced hypoglycemia before and after transabdominal vagotomy plus posterior gastroenterostomy

Time	Volume	Free acid	Total acid	Remarks
	ml.	*C.U.*	*C.U.*	
Three days before operation				
7:20–7:30 a.m.	20	0	16	7:30 20 units insulin given subcutaneously
7:40	30	0	14	
7:50	22	0	17	
8:00	15	0	21	
8:10	10	0	20	
8:20	8	0	15	
8:30	50	17	40	
8:40	64	39	56	8:40 Feels hungry and listless
8:50	39	55	70	
9:00	60	55	69	8:50 Blood sugar 14 mgm. per cent
9:10	60	48	59	
9:20	62	60	72	9:00 Feels weak
9:30	53	42	58	
Ten days after operation				
7:00–7:10 a.m.	6	0	12	
7:20	8	0	6	7:30 Fasting blood sugar 71 mgm. per cent
7:30	3	0	9	
7:40	4	0	9	7:30 20 units insulin given subcutaneously
7:50	14	0	6	
8:00	7	0	10	
8:10	2	0	8	
8:20	1	0	9	
8:30	6	0	21	
8:40	4	0	8	
8:50	4	0	14	
9:00	1	0	9	9:00 Blood sugar 23 mgm. per cent
9:10	1	0	9	
9:20	1	0	17	
9:30	1	0	15	

some cases. Therefore, it was arbitrarily decided to call the response positive only in those cases in which the total acidity increased following insulin administration by more than 20 clinical units over its control values. Using this criterion, 5 of the 22 ulcer patients tested failed to respond preoperatively by increased acidity of the gastric aspirate to 1 or 2 insulin tests. Following vagotomy, 18 of 21 patients tested failed to respond. No patient who failed to respond before operation gave a positive response after operation.

3. *The sham meal test*

The protocols of representative sham meal tests are presented in Tables X to XII. Here again the volumes of the 10-minute samples were so variable that they were considered to be meaningless. Typically a marked increase in the free and total acidity of the gastric aspirate began within 10 to 20 minutes of the mastication of the sham meal and continued for over an hour. Again it was decided to designate a response as positive if the total acidity increased by 20 clinical units or more over its control value. Fifteen of 20 ulcer patients tested before operation gave a positive response to the sham meal. Following vagotomy only 2 of 22 patients tested gave a positive response. All patients who failed to respond to the sham meal before operation also failed to respond postoperatively.

4. *Gastric motility*

Thirteen of the 23 ulcer patients whose response to insulin and the sham meal are reported in this paper underwent a gastric motility test before and after vagotomy. The results were much the same in every case. Two representative tracings are presented in Figures 1 and 2. All 13 patients

TABLE X

Gastric secretory response of duodenal ulcer patient (T. M.) to sham meal before and after transthoracic section of the vagus nerves to the stomach

Time	Volume	Free acid	Total acid	Remarks
	ml.	*C.U.*	*C.U.*	
Six days before operation				
7:30–7:40 a.m.	4	67	84	
7:50	14	69	84	
8:00	2	29	45	8:00 Sham meal begun
8:10	25	46	63	
8:20	20	97	111	8:20 Sham meal completed
8:30	18	76	93	
8:40	13	100	114	
8:50	13	102	117	
9:00	19	98	113	
9:10	7	92	109	
9:20	19	85	100	
9:30	9	69	87	
Ten days after operation				
7:00–7:10 a.m.	7	19	42	
7:20	14	27	51	7:20 Sham meal begun
7:30	12	25	56	
7:40	11	22	57	7:40 Sham meal completed
7:50	4	28	66	
8:00	9	18	60	
8:10	2	30	54	
8:20	12	28	53	
8:30	8	21	53	
8:40	2	28	58	
8:50	8	25	58	
9:00	7	21	58	

TABLE XI

Gastric secretory response of duodenal ulcer patient (I. C.) to sham meal before and after transabdominal section of the vagus nerves plus posterior gastroenterostomy

Time	Volume	Free acid	Total acid	Remarks
	ml.	*C.U.*	*C.U.*	
Two days before operation				
8:00–8:10 a.m.	18	0	14	
8:20	21	14	38	
8:30	21	49	60	8:30 Sham meal begun
8:40	30	70	80	8:40 Sham meal completed
8:50	60	80	102	
9:00	40	97	105	
9:10	20	70	94	
9:20	27	63	71	
9:30	20	74	85	
9:40	20	68	80	
9:50	20	67	80	
10:00	32	58	62	
Seven days after operation				
8:00–8:10 a.m.	8	8	33	
8:20	5	8	27	
8:30	6	15	31	
8:40	7	14	29	
8:50	6	0	24	
9:00	7	10	25	9:00 Sham meal begun
9:10	11	0	30	9:10 Sham meal ended
9:20	7	5	34	
9:30	5	19	38	All samples bile stained
9:40	4	20	38	
9:50	10	0	30	
10:00	9	5	32	

tested showed a basal "tonus" rhythm with a frequency of 2 to 3 per minute (wave duration of 20 to 30 seconds). This was unaltered by vagotomy.

All 13 also showed larger "hunger contraction" waves, as described by Carlson (8), sometimes coming irregularly and sometimes in series, each wave having a duration of roughly 60 seconds. In 12 of these 13 patients these hunger contractions were absent 10 to 14 days following vagus section. In the other patient (J. G.) they were present, but much weaker and more irregular than before operation. The preoperative hunger contraction waves were stronger and more frequent in patients whose ulcers were complicated by a degree of pyloric obstruction than in those without obstruction. There is evidence that these hunger contractions return within several months following vagotomy. They were present and normal in appearance in the case of J. G., when tested 3 months following vagotomy. Another patient (N. W.) had no such waves 10 days following transthoracic vagotomy, but had normal contraction waves 8 days following posterior gastroenterostomy for pyloric obstruction 1½ months later. A third patient (N. K.) had normal rushes of hunger contractions 11 months following transthoracic vagotomy.

In all patients tested, distention of the stomach by the inlying balloon was felt as a vague epigastric pressure or gas pain, and there was no appreciable difference in the quality of the sensation nor its threshold after vagotomy. Five of the 13 patients upon whom motility studies were done were sufficiently introspective accurately to identify preoperatively the hunger contraction waves, describing the sensation as a "pulling in the stomach," a "feeling of suction," and as "hunger pangs." Within the first 2 weeks following vagotomy none of these had sufficiently good contraction

TABLE XII

Gastric secretory response of duodenal ulcer patient (J. A.) to sham meal before and after transabdominal section of the vagus nerves plus subtotal gastric resection

Time	Volume	Free acid	Total acid	Remarks
	ml.	*C.U.*	*C.U.*	
Eleven days before operation				
8:00–8:10 a.m.	34	0	11	
8:20	28	22	39	8:20 Sham meal begun
8:30	11	41	74	
8:40	10	17	69	8:45 Sham meal completed
8:50	7	20	65	
9:00	13	0	49	
9:10	8	33	92	
9:20	9	55	109	
9:30	23	60	98	
9:40	15	56	88	
9:50	42	45	78	
Twelve days after operation				
7:30–7:40 a.m.	5	0	38	
7:50	25	0	26	
8:00	5	0	17	8:00 Sham meal begun
8:10	11	0	23	
8:20	9	0	33	8:20 Sham meal completed
8:30	6	0	27	
8:40	13	0	24	
8:50	6	0	17	
9:00	4	0	19	
9:10	10	0	18	
9:20	6	0	15	
9:30	5	0	19	
9:40	2	0	15	
9:50	6	0	17	
10:00	20	0	23	

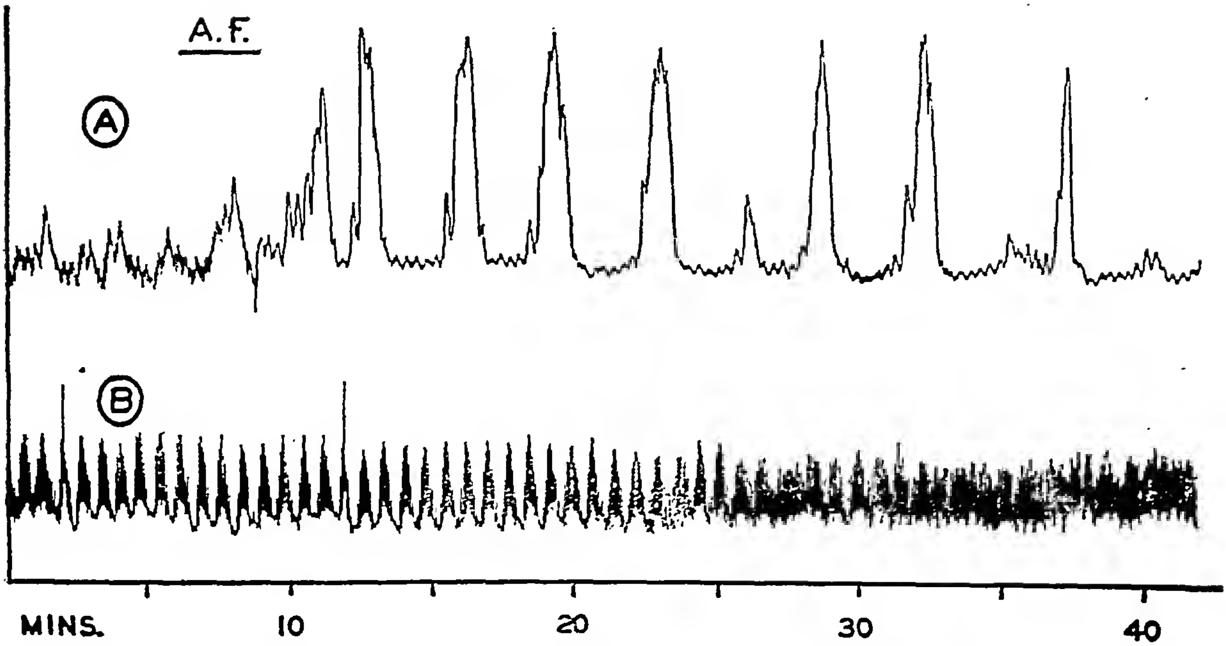

FIG. 1. TRACINGS SHOWING THE MOTILITY OF THE FASTING STOMACH OF A PATIENT (A. F.) WITH A DUODENAL ULCER 2 DAYS BEFORE (A) AND 8 DAYS AFTER (B) SUBDIAPHRAGMATIC VAGOTOMY PLUS POSTERIOR GASTROENTEROSTOMY

waves to give a fair test for postoperative sensation of this sort.

DISCUSSION

During the past 3 years the results of section of the vagus nerves for refractory peptic ulcer have been excellent, both in our hands and those of others (4, 5, 9). Ulcer pain has been absent following vagotomy, but the fact that in a few instances it has occurred briefly makes it unlikely that pain of this sort is mediated via the vagi. Several of our patients (J. G. and N. W.) have suffered rather marked gastric retention following vagotomy, as evidenced by distention, belching of foul gas, and anorexia, and as determined by x-ray examination. This was relieved after gastroenterostomy. In several other patients milder symptoms of this sort, present during the first 2 to 4 weeks following vagotomy, have cleared spontaneously without any additional operative procedure. The initial hypomotility of the stomach after vagotomy will combine with pyloric obstruction, in cases where it is present, to produce decreased gastric emptying. As a result, it is necessary to combine vagus section with gastroenterostomy in cases which show an appreciable amount of pyloric obstruction before operation. It is also imperative to keep an indwelling suction tube in the stomach for the first 2 to 4 days after vagotomy to prevent distention and possible acute dilatation of the atonic viscus.

The postoperative tendency toward loose bowels, which has been an unexpected feature in the postoperative course of 15 of the 42 patients presented here, remains unexplained. On theoretical grounds, parasympathetic denervation of the gastrointestinal tract down to the mid-colon would seem more likely to result in constipation.

The operation of vagus section for peptic ulcers is predicated upon the theory that an abnormally increased interdigestive secretion of gastric acid and possibly pepsin, mediated by impulses proceeding down the vagus nerves, is a major factor in the pathogenesis of such ulcers. Cushing in 1932 (10) reported a group of cases in which lesions in the midbrain resulted in fatal ulceration of the upper gastrointestinal tract. Under experimental conditions typical peptic ulcers can be produced by artificially induced hypersecretion of gastric juice (11) and by permitting the gastric juice to act on the intestinal mucosa without being neutralized by duodenal secretions (12 to 14). It

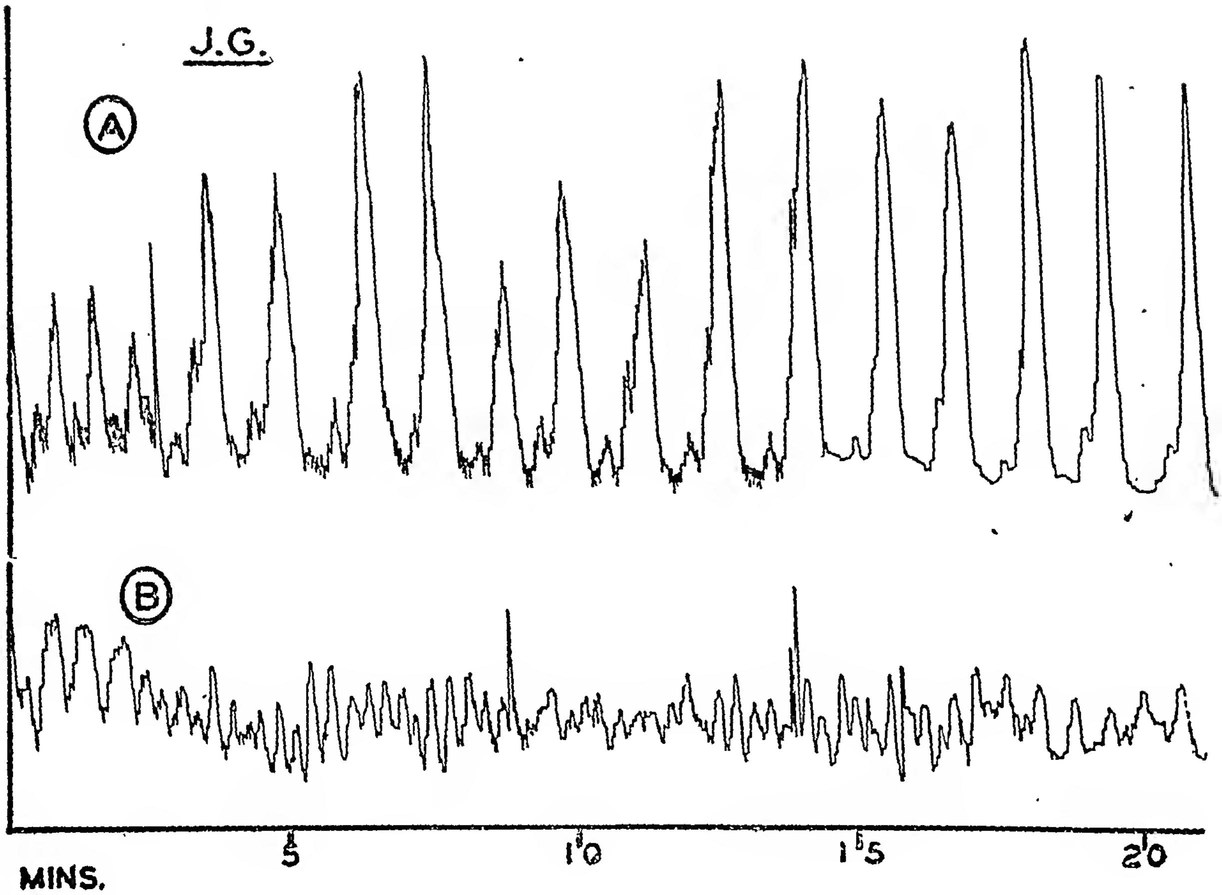

FIG. 2. TRACINGS SHOWING THE MOTILITY OF THE FASTING STOMACH OF A PATIENT (J. G.) WITH A DUODENAL ULCER 1 DAY BEFORE (A) AND 9 DAYS AFTER (B) SUBDIAPHRAGMATIC SECTION OF THE VAGUS NERVES TO THE STOMACH

is therefore important to determine whether or not ulcer patients do have an abnormally increased interdigestive gastric secretion and whether their secretion is reduced following section of the vagus nerves. The continuous nocturnal gastric aspirations reported here represent an effort to answer these questions.

Night secretion tests on patients as performed by the technic described in this paper have their limitations and must be interpreted cautiously. Great care must be taken to ensure that the Levine tube does not become plugged or kinked in the stomach during the night, resulting in a volume of aspirate less than would be obtained under ideal conditions. There is a consistent alteration in character of the aspirate following vagotomy, in that it becomes more mucoid in nature. The increased viscosity may tend to reduce the volume of juice aspirated below its correct value.

There is an inevitable amount of regurgitation of duodenal juice into the stomach in most cases, probably more marked in those patients with gastroenterostomies or gastric resections. This might be expected to alter somewhat the volume of aspirate but should not alter the calculated total HCl output. There is also undoubtedly some loss of gastric juice through the pylorus and this should be decreased in the presence of pyloric obstruction. On comparing Tables IV and V it may be seen that the actual volumes obtained in 18 cases with pyloric obstruction were somewhat elevated above those obtained in 16 cases without appreciable obstruction, but that the free and total acidities were not greatly different in the two groups. Finally, it must be recognized that the non-ulcer patients considered in Tables I to III do not constitute an ideal control group because of the presence of pathology other than ulcer.

Despite these limitations the results summarized in Tables I to VI are sufficiently consistent and the differences sufficiently great to state unequivocally that the continuous night secretion of gastric juice was markedly greater in the ulcer patients than in those without ulcers, both in volume and acidity.

This is particularly emphasized if the calculated total acid output or volume of parietal cell secretion is considered. These conclusions are at variance with those of Sandweiss *et al* (15) whose studies caused them to conclude that the "nocturnal volume of gastric juice in patients with duodenal ulcer (as obtained by continuous suction) is on the average not greater than that of normal subjects of the same age and sex." However, their patients experienced only mild ulcer distress and their ulcer disease was neither so active, so prolonged, so refractory to medical management, nor subject to so many complications as the disease in our series. Even so, they did note hypersecretion of HCl in several of their ulcer patients, and these were, interestingly enough, patients presenting more or less intractable ulcers clinically. In our experience interdigestive gastric secretion studies have constituted a logical and satisfying corroboration to the excellent clinical results of vagus section.

The insulin test was proposed by Ihre (16), Babkin (17), and Hollander (18) as a method of investigating the integrity and function of the vagus nerves to the stomach. Gastric secretion is stimulated as a result of the hypoglycemia induced by insulin (19, 20) and this response is abolished by vagus section (21, 22). In the tests presented in this paper the increase in acidity of the gastric juice occurred at roughly the same time as the maximum fall in blood sugar. In a study of 17 ulcer patients, Thornton, Storer and Dragstedt (5) found that 15 gave a positive response to insulin hypoglycemia before vagotomy and that only 1 of these responded after vagotomy. The results obtained in the present group of 23 additional ulcer patients are roughly the same, a few failing to respond in 1 or 2 insulin tests before operation. Three of our patients gave a definite positive response following operation. A fourth (J. G.), though showing a negative response 2 weeks following vagus section, had a positive response to both the insulin and sham meal test 3 months following vagotomy. It should be noted that all of these 4 patients underwent vagotomy via the abdominal route, a technically more difficult approach to obtain complete bilateral vagus section than through the thorax. Their postoperative response to insulin may be indicative of residual vagus innervation of the stomach. Another patient (N. K.), on the other hand, still showed a negative response to insulin 11 months after vagotomy. We were unable to note any appreciable difference in the response to insulin between our ulcer patients and 6 normal young adults tested in the same manner.

The sham meal test elicits a gastric response which is likewise dependent upon an intact vagal innervation of the stomach. Pavlov showed in dogs (23) that the gastric secretory response to sham feeding and to conditioned reflexes is abolished by vagus section. Necheles and Maskin (24) studied the response of normal and ulcer patients to sham feeding and found little difference between the two groups. A previous attempt on a group of 10 of our patients (5) yielded positive responses before operation in only 3 cases, after operation in none.

Some care must be taken in performing the sham meal test if best results are to be obtained. The inlying Levine tube is naturally distracting to the patient. Positive results were obtained much more consistently in those ulcer patients who had had long experience with gastric intubation than in those unused to the procedure. A positive response was often obtained on a second test, when it had been absent on the first. It is probable that a positive response could finally be obtained in almost every patient if a sufficient numbr of tests were done. Every effort must be made to present the patient with an appetizing sham meal and to shield him from the sight of the expectorated food. If the patient swallows at all during the test, the test is invalidated due to the stimulating effect on secretion of the food itself in the stomach. The response to the sham meal constitutes a conditioned as well as an unconditioned reflex, and psychic inhibition of the normal response occurs easily.

Out of the 20 patients upon whom sham meal tests were done before vagotomy and whose data are summarized in Table XIII, 15, gave a positive response to at least one of 1 or 2 sham meal tests. Following vagotomy, only 2 of 22 gave positive responses. This again may represent residual vagus innervation to the stomach in these cases. The sham meal test and the insulin test appear to be about equally reliable criteria of the integrity of the vagi, and they supplement each other in a useful manner.

TABLE XIII

The secretory response of the stomach to insulin hypoglycemia and to sham feeding before and after section of the vagus nerves to the stomach in patients with duodenal ulcers

Patient	Operation	Preop. response to insulin	Postop. response to insulin	Preop. response to sham meal	Postop. response to sham meal
1. T. M.	Transthoracic vagotomy	+	−	+	−
2. J. S.	Transthoracic vagotomy	−	−		
3. F. V.	Transthoracic vagotomy	+	−	+	−
4. J. D.	Transthoracic vagotomy	+	−	+	−
5. E. K.	Transthoracic vagotomy	−	−	−	−
6. J. M.	Transthoracic vagotomy	+	−	−	−
7. M. H.	Transabd. vagotomy	+	+	+	+
8. J. V.	Transth. vag. (jejunal ulcer)	+		+	−
9. A. W.	Transth. vag. (jejunal ulcer)	+	−	+	−
10. G. H.	Transabd. vag. plus PGE	+	−	+	−
11. J. G.	Transabd. vag. plus PGE	+	−	+	−
12. T. B.	Transabd. vag. plus PGE	+	−	−	−
13. N. W.	Transabd. vag. plus PGE	+	−	+	−
14. L. N.	Transabd. vag. plus PGE	+	+		−
15. A. F.	Transabd. vag. plus PGE	−	−	+	+
16. J. O.	Transabd. vag. plus PGE	+	−	−	−
17. T. B.	Transabd vag. plus PGE		−		−
18. F. H.	Transabd vag. plus PGE	+	−	+	−
19. I. C.	Transabd. vag. plus PGE	+	−	+	−
20. F. L.	Transabd. vag. plus PGE	−		+	−
21. S. N.	Transabd. vag. plus PGE	+	−	+	−
22. E. L.	Transabd. vag. plus PGE	+	+	−	−
23. J. A.	Transabd. vag., gastric resection	−	−	+	−

Note: Results recorded above are derived from 1 or 2 tests of each sort performed before operation and repeated within 2 weeks after operation.

The gastric secretory response to insulin and the sham meal are thus mediated via the vagus nerves, should be absent after complete vagotomy, and is to be sharply differentiated from the response to histamine and caffeine which has been shown (5) to be little altered by vagotomy.

The results presented here regarding gastric motility and sensation in 13 ulcer patients subjected to vagotomy agree with those obtained previously in this clinic in a study of 8 earlier cases and are in accordance with the findings of Moore *et al* (9). Vagus section does not seem to impair the sensation resulting from gastric distention, nor the sensations of nausea and appetite. Ulcer pain can be felt following vagotomy. We have not yet been able to determine whether typical hunger pang sensations that can be correlated with gastric hunger contractions persist following vagus section.

SUMMARY

1. The continuous night secretion of gastric juice in the empty stomach of patients with benign ulcer is, as a rule, greater in volume and acidity than that of patients without such ulcers. It is usually markedly reduced following section of the vagus nerves to the stomach.

2. The secretory response of the fasting stomach of patients with benign ulcer to the stimulation of insulin hypoglycemia and of a sham meal is abolished by vagus section.

3. The motility of the empty stomach of patients with benign ulcer is, as a rule, markedly reduced for at least 2 weeks following section of the vagus nerves to the stomach. It returns toward normal limits within a period of 1 to 3 months.

4. Following section of the vagus nerves to the stomach, our ulcer patients have shown healing of their ulcers on x-ray examination and, with rare exceptions, they have experienced immediate and persistent relief from ulcer pain and freedom from subsequent development of hemorrhage, perforation, or pyloric stenosis.

BIBLIOGRAPHY

1. Dragstedt, L. R., and Owens, F. M., Jr., Supradiaphragmatic section of vagus nerves in treatment of duodenal ulcer. Proc. Soc. Exper. Biol. and Med., 1943, 53, 152.
2. Dragstedt, L. R., Palmer, W. L., Schafer, P. W., and Hodges, P. C., Supra-diaphragmatic section of the vagus nerves in the treatment of duodenal and gastric ulcers. Gastroenterology, 1944, 3, 450.
3. Dragstedt, L. R., and Schafer, P. W., Removal of the vagus innervation of the stomach in gastroduodenal ulcer. Surgery, 1945, 17, 742.
4. Dragstedt, L. R., Vagotomy for gastroduodenal ulcer. Ann. Surg., 1945, 122, 973.
5. Thornton, T. F., Jr., Storer, E. H., and Dragstedt, L. R., Supradiaphragmatic section of the vagus

nerves; effect on gastric secretion and motility in patients with peptic ulcer. J. A. M. A., 1946, **130**, 764.
6. Cole, W., Personal communication.
7. Hollander, F., and Cowgill, G. R., Studies in gastric secretion. I. Gastric juice of constant acidity. J. Biol. Chem., 1931, **91**, 151.
8. Carlson, A. J., The Control of Hunger in Health and Disease. University of Chicago Press, Chicago, 1916.
9. Moore, F. D., Chapman, W. P., Schulz, M. D., and Jones, C. M., Trans-diaphragmatic resection of the vagus nerves for peptic ulcer. New England J. Med., 1946, **234**, 241.
10. Cushing, H., Peptic ulcers and interbrain. Surg., Gynec., and Obst., 1932, **55**, 1.
11. Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., Experimental production of gastric and duodenal ulcers in laboratory animals by intramuscular injection of histamine in beeswax. Surg., Gynec., and Obst., 1942, **75**, 170.
12. Mann., F. C., and Williamson, C. S., Experimental production of peptic ulcer. Ann. Surg., 1923, **77**, 409.
13. Matthews, W. B., and Dragstedt, L. R., Etiology of gastric and duodenal ulcer; experimental studies. Surg., Gynec., and Obst., 1932, **55**, 265.
14. Elman, R., and Hartmann, A. F., Spontaneous peptic ulcers of duodenum after continued loss of total pancreatic juice. Arch. Surg., 1931, **23**, 1030.
15. Sandweiss, D. J., Sugarman, M. H., Podolsky, H. M., and Friedman, M. H. F., Nocturnal gastric secretion in duodenal ulcer. J. A. M. A., 1946, **130**, 258.
16. Ihre, B. J. E., Human Gastric Secretion. Oxford University Press, London, 1939.
17. Babkin, B. P., Secretory Mechanism of the Digestive Glands. Paul B. Hoeber, Inc., New York, 1944.
18. Hollander, F., In discussion of paper on vagus section for ulcers. Gastroenterology, 1944, **3**, 466.
19. Roholm, K., Clinical investigations into effect of intravenous injection; gastric secretion in normal individuals. Acta med. Scandinav., 1930, **73**, 472.
20. Kalk, H., and Meyer, P. F., Blutzuckerspiegel und Magensekretion. Ztschr. f. klin. Med., 1932, **120**, 692.
21. La Barre, J., and de Cespédès, C., Les variations de le sécrétion gastrique au cours de l'hypoglycémic insulinique. Compt. rend. Soc. de biol., 1931, **106**, 480.
22. Okada, S., Kuramochi, K., Tsukahara, T., and Ooinoue, T., Pancreatic function: humoroneural regulation of gastric, pancreatic and biliary secretions. Arch. Int. Med., 1929, **43**, 446.
23. Pavlov, I. P., The Work of the Digestive Glands. Charles Griffen, London, 1910.
24. Necheles, H., and Maskin, M. H., Studies on constitution and peptic ulcer; appetite secretion in normal persons and in ulcer patients. Am. J. Digest. Dis. and Nutrition, 1936, **3**, 90.

METABOLIC ALTERATIONS FOLLOWING THERMAL BURNS. VII. EFFECT OF FORCE-FEEDING, METHIONINE, AND TESTOSTERONE PROPIONATE ON NITROGEN BALANCE IN EXPERIMENTAL BURNS[1]

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Previous work (1) demonstrated that following an experimental burn animals were in negative nitrogen balance on a caloric and protein intake that had been sufficient to maintain nitrogen equilibrium during several control periods prior to burning. The total circulating plasma proteins in these animals remained the same, or in some instances increased slightly, while the concentration and total amount of albumin decreased (2). It has been well established (3 to 6) that patients suffering from burns show a nitrogen deficit and a decrease in the plasma protein concentration. Since it has been suggested that the prevention or alleviation of the nitrogen loss would improve the status of the patient it seemed important to attempt to do this by increasing the protein intake or by reducing the protein catabolism through the administration of testosterone propionate, or methionine. It has been proposed that if force-feeding of protein is employed that such feeding should be started at the earliest possible moment (6, 7). By means of increasing the nitrogen intake in a group of patients, the negative nitrogen balance was prevented or diminished, but in most instances when the very high diets were given early, they were poorly tolerated (8). In order to have a better understanding of the importance of securing a positive nitrogen balance at an early stage, and ways of attaining it, dogs were treated shortly after being burned, by force-feeding or by a normal diet plus methionine or intramuscular injections of testosterone propionate.

EXPERIMENTAL

Selection of the animals, diet, and methods of analyses as well as method of burning have been previously described (1). Four animals were used for the force-feeding experiments and 2 each for the testosterone and methionine studies. The first 2 animals in the force-fed groups (Nos. 10 and 11) were given a mixture of Nutramigen and Amigen[2] following the burn, in an amount sufficient to triple the nitrogen and double the caloric intake they had received previous to the burn. The other animals (Nos. 12 and 13) in the force-fed group following the burn were fed a mixture of Amigen and Aminoids[3] which provided a nitrogen intake approximately 3 times the amount they had taken during the control period. The caloric intake in this latter group was not materially altered. The food was mixed in a Waring Blendor with a measured amount of water and fed by stomach tube in 3 divided doses. The first feeding was administered a few hours following the burn. Two animals (Nos. 14 and 15) were maintained on the same diet they had received previous to the burn and were given intramuscular injections of 25 mgm. of testosterone propionate[4] daily for 15 days. Two other animals (Nos. 16 and 17) following the burn were given the regular diet plus methionine[5] in an amount equal to 1.5 per cent of the diet.

RESULTS

The data on 3 of the 4 force-fed animals (Nos. 11, 12 13) are shown in Table I. The nitrogen deficit was reduced over that of the previously reported normally fed dogs (1), but the institution of early force-feeding was poorly tolerated. No collections were obtained on dog No. 10 of the force-fed group because of marked vomiting and diarrhea, and the clinical condition of this animal was such that it was sacrificed on the sixth day following the burn. The food intake was reduced for dog No. 11 and a 3-day urine sample was obtained from the 4th to the 7th day. The results indicate that the animal was close to nitrogen balance dur-

[1] The work described in this paper was done in part under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Wayne University College of Medicine. It was supported in part by a grant from the Theodore A. McGraw Fund.

[2] Kindly supplied by Mead Johnson and Co., Evansville, Ind.

[3] Kindly supplied by the Arlington Chemical Co., Yonkers, N. Y.

[4] Perandren, kindly supplied by the Ciba Pharmaceutical Products, Inc.

[5] Kindly supplied by Merck & Co., Inc. Rahway, N. J.

TABLE I

The effect of force-feeding on the nitrogen balance following an experimental burn

Dog	Period	No. of days in period	Ave. N intake per day	Ave. nitrogen output per day: Total urinary N	Fecal N	Total N	Ave. N balance	Caloric intake	Body wt.*
11	1	4	9.76	7.87			1.89†	850	15.2
	2	5	9.76	7.00			2.76†	850	15.3
	3	6	9.67	8.41			1.26†	850	15.4
	Burned 20 per cent								
	4	4	26.27¶	discarded‡				1,799	15.9
	5	3	21.40¶	19.21			1.49†	1,439	16.2
12	1	6	8.90	6.79	.86	7.65	1.25	750	13.7
	2	3	8.90	7.41	.23	7.64	1.26	750	13.7
	3	5	25.70**	discarded‡					
	4	4	8.90	8.61	.89	9.50	−0.60	750	14.2
	5	5	8.90	7.50	.79	8.29	0.61	750	14.2
	6	5	8.90	7.77	.65	8.42	0.48	750	14.2
	7	4	8.90	7.54	.65§	8.19	0.71	750	14.2
	8	5	8.90	7.03	.42	7.45	1.45	750	14.2
	9	4	8.87	7.07	.47	7.54	1.33	750	14.3
	Burned 20 per cent								
	10	5	25.70††			21.56‖	4.14	925	15.5
	11	5	25.70††			27.14‖	−1.44	925	13.8
	12	2	13.27	sample lost				1,535	14.0
	13	5	8.87	9.17	.74	9.91	−1.04	925	12.7
	14	5	21.28	14.01	2.11	16.12	5.16	1,818	14.2
	15	6	14.47	11.78	.85	12.63	1.84	1,041	14.4
13	1	4	8.90	7.14	.61	7.75	1.15	750	13.8
	2	5	8.90	6.78	.35	7.13	1.77	750	13.7
	3	5	25.70**			20.43‖	5.27	1,725	15.0
	4	4	8.90	9.99	.61	10.60	−1.70	750	14.4
	5	5	8.90	8.88	.37	9.25	−0.35	750	14.3
	6	5	8.90	7.56	.58	8.14	0.76	750	14.5
	7	4	8.90	7.49	.50	7.99	0.91	750	14.2
	8	5	8.89	7.30	.41	7.71	1.18	750	14.2
	9	4	8.87	6.93	.32	7.25	1.62	750	14.3
	Burned 20 per cent								
	10	5	25.70††			22.19‖	3.51	925	14.2
	11	5	25.70††			28.21‖	−2.51	925	13.8
	12	7	1.84	5.93	.46	6.39	−4.55	156	11.2
	13	5	7.10	7.59	.48	8.07	−0.97	605	11.0
	14	6	8.81	5.11	.47	5.58	3.23	750	11.5

* Weight at end of period.
† Intake minus urinary nitrogen output.
‡ Discarded because urine contaminated with vomitus and feces.
§ Average fecal nitrogen output.
‖ Includes urine, vomitus, and fecal nitrogen.
¶ 300 grams Nutramigen and 200 grams Amigen in 550 ml. of water 1st day; then reduced to 200 grams Nutramigen, 133 grams Amigen in 360 ml. of water.
** 240 grams Nutramigen and 160 grams Amigen in 440 ml. of water.
†† 140 grams Amigen, 100 grams Aminoids, 16 grams yeast, 5.4 grams salt and 1.5 grams cod liver oil in 300 ml. of water.

ing this time. However, the animal rapidly grew worse and was sacrificed on the 12th day.

Since the response to the early high food intake in these 2 animals was unsatisfactory, it seemed desirable to study the effect of such a regime on 2 normal animals (Nos. 12 and 13). It was found that this high intake (at least in the form given) was also poorly tolerated by the normal dog, al-

TABLE II

The effect of daily injections of 25 mgm. testosterone propionate on the nitrogen balance following experimental burns

Dog	Period	No. of days in period	Ave. N intake per day	Ave. nitrogen output per day			Ave. N balance	Body wt.*
				Total urinary N	Fecal N	Total N		
14	1	5	8.37	6.05	.66	6.71	1.66	14.3
	2	5	8.37	7.17	.57	7.74	0.67	13.8
	3	5	8.41	6.75	.63	7.38	1.03	13.7
	Burned 20 per cent							
	4	5	8.45‡	9.50	.55	10.05	−1.55	13.8
	5	6	8.59‡	9.70	.60	10.30	−1.71	12.8
	6	4	5.57‡	8.21	.60†	8.81	−3.24	—
15	1	5	10.71	8.46	.74	9.20	1.51	16.4
	2	5	10.77	9.46	1.01	10.47	0.30	16.8
	3	5	10.77	9.74	0.74	10.48	0.29	16.9
	Burned 20 per cent							
	4	5	10.82‡	12.17	0.66	12.83	−2.02	16.9
	5	6	11.00‡	11.22	0.64	11.86	−0.86	16.7
	6	4	11.00‡	9.63	1.00	10.63	0.37	16.2
	7	5	10.43	9.57	0.37	9.94	0.49	15.5
	8	5	10.43	7.94	1.00	8.94	1.49	15.0
	9	5	10.53	7.92	0.78	8.70	1.83	15.2
	10	7	10.56	7.59	0.62	8.21	2.35	15.0
	11	7	10.58‡	7.43	0.78	8.21	2.36	15.8

* Weight at end of period.
† Average fecal nitrogen output.
‡ 25 mgm. testosterone propionate intramuscular daily.

though the symptoms were not so severe as those exhibited by the burned animals. The total nitrogen output (urine, feces, and vomitus) was analyzed for dog No. 13 for the 5-day period and in spite of loss through vomiting, the positive nitrogen balance (period 3) was markedly increased over the control periods. A gain in weight occurred, which was thought to be partly due to water retention. After allowing several weeks for these animals to return to normal, they were burned and then force-fed a diet containing 3 times the nitrogen content of the control diet, but with little change in caloric content. Clinically the animals seemed better than the first 2 dogs (Nos. 10 and 11) but their course was far from satisfactory. The total nitrogen output in these dogs was analyzed during two 5-day periods of force-feeding. Both animals were in marked positive nitrogen balance during the first 5 days following the burn, but in negative balance the second 5 days. Following the 10 days of force-feeding, the animals were given the regular diet *ad libitum*. Dog No. 12 consumed large amounts of food, and nitrogen retention was fairly marked. A gain in weight without evidence of edema also occurred. The other animal ate poorly and did not regain his control weight.

The results on the 2 testosterone-treated dogs (Nos. 14 and 15) are shown in Table II. Dog No. 14 showed a slight skin disorder during the control period which did not seem significant. However, 10 days following the burn, it became worse and there was every indication that the results obtained during the last 5 to 7 days were complicated by this factor. The dog started to refuse food during the sixth period and the experiment was discontinued on the fifteenth day post burn.

The other animal (No. 15) showed only a slight loss of nitrogen after the fifth day and was in positive balance after the tenth day. This was an improvement over the 4 control dogs which were in negative balance for 15 days following the burn.

The nitrogen balance studies on the methionine-treated dogs (Nos. 16 and 17) are shown in Table III. The animals refused the food with the methionine supplement; so it was given by stomach

TABLE III

The effect of methionine supplement on the nitrogen deficit following experimental burns

Dog	Period	No. of days in period	Ave. N intake per day	Total urinary N	Ave. N balance*	Body wt.†
16	1	4	13.70	9.80	3.90	20.2
	2	5	13.92	10.42	3.21	20.6
	3	7	13.92	11.63	2.00	20.3
	Burned 20 per cent					
	4	5	14.23‡	16.33	− 2.19	20.0
	5	2	0	15.45	−15.45	—
17	1	4	14.27	11.78	2.49	21.8
	2	5	14.50	11.40	3.10	21.8
	3	7	14.50	11.07	3.43	22.0
	Burned 20 per cent					
	4	5	14.83‡	17.65	−2.95	21.8
	5	4	2.50	10.82	−8.32	20.0

* Intake minus urinary N output.
† Weight at end of period.
‡ Supplemented with 1.5 per cent methionine daily (dog 16, 3.60 grams; dog 17, 3.75 grams).

tube. One of the animals (No. 16) started to vomit on the third day and the course of both animals was poor. Since the supplement in no way seemed to improve the condition over the untreated animals, the methionine was discontinued after the fifth day and the regular diet resumed. Dog No. 16 refused all food and died on the seventh day post burn. The other animal died on the tenth day.

DISCUSSION

No conclusions can be drawn concerning the nitrogen balance studies in the first 2 force-fed dogs (Nos. 10 and 11) which were given the high protein and high caloric diet, but from the 1 collection period for dog No. 11, it would appear that the nitrogen loss was reduced. The nitrogen balance of the other force-fed dogs (Nos. 12 and 13), which were given triple the nitrogen intake but almost the same caloric intake, was negative during the second 5-day period in spite of the high nitrogen intake. The course of the animals was certainly in no way improved by the positive balance during the first 5 days. Cuthbertson (9) noted in his work on fracture patients that high caloric and protein diets (as much as 231 grams protein and 4,100 calories) failed to eliminate the negative nitrogen balance during the height of the catabolic phase. He first observed a period of retention, then a period of loss which was followed later by a period of retention. This pattern is similar to that noted in our animals. Howard, Winternitz, Parson, Bigham and Eisenberg (10) found that fracture patients on a low protein, low caloric diet lost no more nitrogen than those on a higher nitrogen and caloric intake. However, their high diets contained only 15 grams of nitrogen and 24 calories per 1.73 sq. m. area and therefore cannot be considered similar to those of Cuthbertson (9).

No attempt was made to force-feed animals with large amounts of their regular diet. It seemed of particular interest to use amino acid preparations since their use has been advocated for increasing the protein intake. It is quite possible that large amounts of native protein may be somewhat better tolerated than the hydrolysates or that the amino acid mixtures if started after the shock phase had subsided and gradually increased might have been beneficial. Free and Leonards (11) found that when large equivalent amounts of nitrogen were taken in the form of meat, blood, and amino acids, the latter form caused gastrointestinal upsets in both subjects. While the form of nitrogen and the fact that it was given in such large amounts may have aggravated the diarrhea and vomiting noted in our animals, the gastrointestinal upset was not the only undesirable effect noted in these experiments.

Plasma protein, albumin, and plasma volume alterations were followed in these animals and will be discussed in another report. However, it can be stated here that the volume changes were more marked and no doubt contributed to the undesirable effects of force-feeding. Since it has been shown (12) that increased intakes of nitrogen are accompanied by an increased intake of water, the imbalance following a burn was, no doubt, accentuated by feeding large amounts of protein. That the force-feeding in itself has an effect on water metabolism is indicated by the studies on the 2 normal animals. During the 5-day period of force-feeding (period 3) previous to the burn, dog No. 13 consumed more than 4 times the amount of water than that drunk on the regular diet and the apparent water retention (intake minus urine output) was doubled over the control periods. Following the burn, all the force-fed animals showed a marked edema and several had generalized muscular twitchings which had been noted by other workers (13, 14) in cases of "water intoxication."

Once the anabolic phase begins (from 15 to 20 days post burn in our animals) large amounts of food can be taken to advantage. During period 14, dog No. 12 voluntarily consumed 21 grams of nitrogen and retained 5 grams per day with a gain in weight of 1.5 kgm. Another animal (not shown in the table) was also given food *ad libitum* from the nineteenth to twenty-fifth day post burn and consumed an average of 33 grams of nitrogen per day with a urinary nitrogen output of 26 grams, and during this time gained 2.3 kgm. Neither of these dogs showed edema or the other undesirable effects seen in the early force-fed dogs.

Since testosterone propionate has been shown to increase nitrogen retention (15), there has been an interest in the use of this hormone to decrease the loss of nitrogen following injury. Its use has

been reported by Howard (16) in a fracture patient in whom it seemed to have some effect in reducing the nitrogen loss. Testosterone propionate was therefore administered to 2 female dogs following a standardized burn. It is difficult to draw conclusions from this work, since only 2 animals were studied, and the results on the one animal were complicated by the skin disorder previously described. Because of comparable results obtained with testosterone propionate in patients (17), it seems that the improvement in the nitrogen balance noted in dog No. 15 might be of some significance. The use and cautions to be exercised in employing testosterone propionate have been brought out in another report (17).

Croft and Peters (18) reported that the addition of methionine reduced the nitrogen loss following burns in rats. They presented the hypothesis that there is a need for methionine following injury and that to cover this requirement, tissue is broken down resulting in a large loss of nitrogen. The methionine supplement administered to the 2 dogs in the present experiment was without effect on the nitrogen balance. While no conclusions can be drawn from this small group, the results are in keeping with the negative findings in a patient treated with this amino acid and with the report of Schenker (19) and Chanutin and Ludewig (20) who noted no improvement in the nitrogen balance with the administration of methionine.

Howard and his associates (10) have pointed out that the nitrogen loss following injury does not occur in malnourished patients and apparently feel that the nitrogen catabolism in healthy injured subjects may be a desirable reaction. There seems to be some question, therefore, whether it is advantageous to attempt to prevent or alleviate the nitrogen loss during the catabolic phase. Cuthbertson, Shaw and Young (21) reported that a crude anterior pituitary extract prevented weight loss and reduced the nitrogen loss in injured rats, but did not increase the rate of restoration of the muscles which atrophied as the result of injury.

These observations force one to question the advisability of attempting to prevent the loss of nitrogen by force-feeding during the first week or 2 following a burn or other severe trauma. It is obvious, however, that if a negative nitrogen balance persists for more than 2 or 3 weeks the patient will suffer from a serious protein depletion. It is important, therefore, to institute adequate feedings as soon as the patient can tolerate them. It is our impression (17) and that of others (22) that during the first 2 days the caloric and protein intake should not be forced. In several very severely burned adult patients we have recently given between 800 and 1,200 calories (mostly carbohydrate) during the first 2 days following the burn. The caloric and protein intake was then increased to 1.6 times the patients' basal caloric requirement and subsequently gradually increased to an intake of 200 grams protein and about 3,000 calories. These patients exhibited a minimum nitrogen loss and seemingly have been in as good a condition as can be expected.

SUMMARY

Four groups of 2 female dogs were fed a standardized diet and, after a burn, given the following treatment:

1. Force-fed a diet containing triple the protein (in the form of amino acids) and twice the caloric intake received during the control periods.
2. Force-fed a diet containing triple the protein, but little change in caloric intake.
3. The regular diet and injections of 25 mgm. testosterone propionate daily.
4. The regular diet plus 1.5 per cent methionine.

The results indicate that force-feeding improved the nitrogen balance, but the treatment was poorly tolerated.

There is some indication that testosterone propionate reduces the nitrogen loss following a burn. Methionine seemed to have no beneficial effect on the nitrogen deficit which occurs after a burn.

BIBLIOGRAPHY

1. Meyer, F. L., Joseph, S., Hirshfeld, J. W., and Abbott, W. E., Metabolic alterations following thermal burns. I. Nitrogen balance in experimental burns. J. Clin. Invest., 1945, 24, 579.
2. Abbott, W. E., Hirshfeld, J. W., and Meyer, F. L., Metabolic alterations following thermal burns. II. Changes in plasma volume and plasma protein in the convalescent phase. Surg., Gynec. and Obst., 1945, 81, 25.
3. Davidson, E. C., Sodium chloride metabolism in cutaneous burns and its possible significance for a rational therapy. Arch. Surg., 1926, 13, 262.

4. Lucido, J., Metabolic and blood chemical changes in a severe burn; a case report. Ann. Surg., 1940, 111, 640.
5. Taylor, F. H. L., Levenson, S. M., Davidson, C. S., Adams, M. A., and MacDonald, H., Abnormal nitrogen metabolism in burns. Science, 1943, 97, 423.
6. Taylor, F. H. L., Levenson, S. M., Davidson, C. S., Browder, N. C., and Lund, C. C., Problems of protein nutrition in burned patients. Ann. Surg., 1943, 118, 215.
7. Harkins, H. N., Cope, O., Evans, E. I., Phillips, R. A., and Richards, D. W., Jr., The fluid and nutritional therapy of burns. Memorandum prepared by a committee appointed by Dr. Alfred Blalock, chairman of the subcommittee on shock. J. A. M. A., 1945, 128, 475.
8. Hirshfeld, J. W., Abbott, W. E., Pilling, M. A., Heller, C. G., Meyer, F. L., Williams, H. H., Richards, A. J., and Obi, R., Metabolic alterations following thermal burns. III. Effect of variations in food intake on nitrogen balance of burned patients. Arch. Surg., 1945, 50, 194.
9. Cuthbertson, D. P., Post-shock metabolic response. (Arris and Gale Lecture.) Lancet, 1942, 1, 433.
10. Howard, J. E., Winternitz, J., Parson, W., Bigham, R. S., and Eisenberg, H., Studies on fracture convalescence. II. The influence of diet on post-traumatic nitrogen deficit exhibited by fracture patients. Bull. Johns Hopkins Hospital, 1944, 75, 209.
11. Free, A. H., and Leonards, J. R., Studies on ingestion of large quantities of protein and amino acids. J. Lab. and Clin. Med., 1944, 29, 963.
12. Gamble, J. L., Putman, M. C., and McKhann, C. F., The optimal water requirement in renal function. I. Measurements of water drinking by rats according to increments of urea and of several salts in the food. Am. J. Physiol., 1929, 88, 571.
13. Trusler, H. M., Egbert, H. L., and Williams, H. S., Burn shock: Question of water intoxication as a complicating factor; blood chemical studies and report of an extensive burn treated by repeated transfusions of blood and blood plasma. J. A. M. A., 1939, 113, 2207.
14. Rowntree, L. G., Water intoxication. Arch. Int. Med., 1923, 32, 157.
15. Kenyon, A. T., Knowlton, K., Sandeford, I., Koch, F. C. and Lotwin, G., A comparative study of the metabolic effects of testosterone propionate in normal men and women and in eunuchoidism. Endocrinology, 1940, 26, 26.
16. Minutes of the conference on metabolic aspects of convalescence including bone and wound healing. New York, Josiah Macy, Jr. Foundation, Fourth Meeting, June 11–12, 1943, p. 74.
17. Abbott, W. E., Hirshfeld, J. W., Williams, H. H., Pilling, M. A., and Meyer, F. L., Metabolic alterations following thermal burns. VI. The effect of altering the nitrogen and caloric intake or of administering testosterone propionate on the nitrogen balance. Surgery, 1946, 20, 284.
18. Croft, P. B., and Peters, R. A., Nitrogen loss after thermal burns, effects of adding protein and methionine to diets of rats. Lancet, 1945, 1, 266.
19. Schenker, V., The characteristic changes in nitrogen metabolism after damage. Proc. Canad. Physiological Soc., 9th annual meeting, Nov. 2–3, 1945.
20. Chanutin, A., and Ludewig, S., Personal communication.
21. Cuthbertson, D. P., Shaw, G. B., and Young, F. C., The anterior pituitary gland and protein metabolism. II. The influence of anterior pituitary extract on rate of wound healing. J. Endocrinol., 1941, 2, 475.
22. Levenson, S. M., Personal communication.

THE BILIARY EXCRETION OF VARIOUS BILE ACIDS BY PATIENTS WITH CHOLEDOCHOSTOMY DRAINAGE, AND THE EFFECT OF ORAL ADMINISTRATION OF DESICCATED BILE OF THE OX AND THE HOG [1]

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Choledochostomy drainage of bile by means of a T-tube frequently is established following surgery of the biliary tract, and often such drainage is maintained for periods ranging from several weeks to several months. During this time only small amounts of bile, usually quite deficient in bile salts (1, 2), enter the intestine. Replacement by oral administration of bile is indicated. A method for large-scale preparation of desiccated bile has been reported, and average analyses of desiccated gallbladder bile of the hog have been presented (3). Such preparations of desiccated bile are being used with success in replacement therapy (4) and for symptomatic treatment of patients with gastrointestinal disorders associated with biliary insufficiency (5).

Bile acids of man, the ox, and the hog consist of a steroid nucleus possessing a carboxyl group which usually is conjugated through amide linkage with glycine or taurine. In bile of man and the ox approximately 50 to 75 per cent of the total quantity of the bile acids [2] is conjugated with glycine and the major portion of the remainder with taurine (6). However, this proportion is somewhat variable, and often a considerable fraction of the bile acids is in the "free" or unconjugated form (7). By contrast, the bile acids of hog bile are conjugated solely with glycine (6, 8). The bile acids of these 3 animals differ also in the nature of the steroid nuclei and in the relative proportions of these bile acids present in the bile. The principal bile acid (steroid nucleus) of hog bile is α-hyodesoxycholic acid (3,6-dihydroxycholanic acid) (8). The principal bile acid in bile of man and the ox is cholic acid (3,7,12-trihydroxycholanic acid), but important amounts of desoxycholic acid (3,12-dihydroxycholanic acid) and chenodesoxycholic acid (3,7-dihydroxycholanic acid) also are present (9, 10). Hyodesoxycholic acid has not been found in bile of man and the ox, and cholic acid is absent from the bile of the hog.

In view of the qualitative and quantitative differences in the bile acid composition of bile of various animals, it is important to have information concerning the utilization of various bile acids as a guide to adequate replacement therapy in human patients with biliary insufficiency. In previous communications (11, 12) we have reported experiments which have demonstrated very efficient enterohepatic circulation of foreign bile acids in animals with experimental biliary fistulae. The present paper is a description of studies on the administration of desiccated bile of the hog and the ox to human patients with choledochostomy drainage.

EXPERIMENTAL

Cholic acid and total dihydroxycholanic acids [3] were determined by methods which we have described previously (12, 13). The method for determining "cholic acid" yields the sum of free cholic acid, glycocholic, and taurocholic acids. Desoxycholic acid was determined by an adaptation of the procedure of Kaziro and Shimada (14). The reaction with benzaldehyde was conducted in 23 N sulfuric acid instead of the more concentrated acid specified by Kaziro and Shimada, thus minimizing interference by chenodesoxycholic acid. In order to attain greater specificity and eliminate interfering substances, the reaction was applied to the dihydroxycholanic acid fraction which was separated from cholic acid and from the monohydroxycholanic acid fraction by the pro-

[1] Aided by a grant from Parke, Davis and Company.

[2] It is customary to refer to the bile "acids" in bile although it is understood that at the pH of bile these acids are almost completely ionized as anions which are matched by sodium, potassium, and calcium cations.

[3] The dihydroxycholanic acids which have been isolated (9, 21) from bile of man are desoxycholic acid and chenodesoxycholic acid. Hyodesoxycholic acid of hog bile also is a dihydroxycholanic acid (8, 15). Our method for the determination of "total dihydroxycholanic acids" (12) yields the *sum* of these 3 acids.

cedure which we have described previously (12). Optical density measurements were made with a filter photometer at a mean wavelength of 620 mμ.

For the determination of hyodesoxycholic acid a sample of bile estimated to contain 25 to 75 mgm. of this acid was extracted and hydrolysed, and the dihydroxycholanic acid fraction was separated by the method previously described (12). The dihydroxycholanic acids, including hyodesoxycholic acids, were oxidized by chromic acid to diketocholanic acids as described previously (12), a proportionately larger amount of chromic acid being required because of the larger sample. The diketocholanic acid fraction was dissolved in glacial acetic acid, and the optical rotation (D line of sodium) was determined in a polarimeter equipped with a micro tube of 1 decimeter length requiring 1 ml. of solution. Then hydrochloric acid was added to the solution to a concentration of 0.5 per cent by volume, and the solution was boiled for 1 hour under reflux. During this process, the 3,6-diketocholanic acid (resulting from the oxidation of hyodesoxycholic acid) underwent rearrangement to 3,6-diketoallocholanic acid. The solution was evaporated to dryness in vacuo; then the residue was dissolved again in glacial acetic acid and brought to a volume equal to that used in the initial polarimetric measurement. The optical rotation of this solution was determined, and the initial quantity of hyodesoxycholic acid in the sample was calculated from the *change* in optical rotation which accompanied the rearrangement of 3,6-diketocholanic acid to 3,6-diketo-allocholanic acid. This method is specific for hyodesoxycholic acid inasmuch as the other diketocholanic acids (resulting from the oxidation of the other dihydroxycholanic acids) do not undergo such rearrangement. Calculations were based upon determinations with standard amounts of hyodesoxycholic acid which were carried through the same procedure, the "unknown" being referred to polarimetric values which were obtained with standards of nearly the same concentration. This method utilizes principles which were described in the studies of Windaus *et al* upon the constitution of hyodesoxycholic acid (8, 15). Following the polarimetric measurements, a portion of the diketocholanic acid fraction was used for determination of the total diketocholanic acids from which the total dihydroxycholanic acids originally present in the sample could be calculated.

Total bile acids were calculated as the sum of cholic acid and the total dihydroxycholanic acids. This calculation of the "total bile acids" left unaccounted the traces of monohydroxycholanic acids and keto bile acids which have been found in bile.

Total conjugated bile acids and tauro-conjugated acids were determined essentially by the method of Schmidt and Dart (16). Glyco-conjugated acids were calculated by difference between the total conjugated and the tauro-conjugated acids.

The patients studied in this report were all treated for obstruction of the common duct.[4] Patients C. D., A. M., and C. M. had institution of drainage of their common duct by means of a T-tube. Patient F. W. was in exceedingly poor shape when operated upon; the gall-bladder was removed and drainage instituted by a large tube placed in the dilated cystic duct. Bile was allowed to flow unimpeded into a sterile bottle with a vent as described by Richey (17), the bottle being placed a few inches below the level of the patient. The few inches which the bile flowed by gravity through a large tube was not considered sufficient to obstruct the flow by causing collapse of the tube.

The collection of bile in these cases could not be considered as complete. However, since there was no impediment offered to the outflow of bile, it is to be presumed that very little bile found its way into the duodenum and that most of it was collected. That this assumption is valid has been corroborated by the fact that hyodesoxycholic acid, a bile acid not found in significant quantities in man, was recovered almost quantitatively through the drainage tube after administration to the patient by mouth. It was not possible to control each study, and the possibility of partial recoveries introduces the probability of error. Those errors which are due to incomplete recoveries would tend to give lower values than actually found. Accordingly, in considering the data presented on recoveries of bile acid, it must be recognized that there is a possibility that they may be somewhat low rather than high.

DISCUSSION OF RESULTS

The excretion of various bile acids in the choledochostomy drainage bile of these cases can be discussed more clearly by dividing the presentation into a section on the excretion in the absence of orally administered animal bile and a section on the excretion during administration of bile. Eleven cases were studied, but this report is confined to a discussion of 5 cases which were investigated in greatest detail and which illustrate the principal observations and conclusions which were derived from the entire series.

(1) *Excretion of bile acids in the absence of orally administered animal bile:* Following cholecystectomy and establishment of choledochostomy drainage of bile, a post-operative period of 10 to 20 days usually elapses before resumption of synthesis of bile acids by patients whose biliary tracts have been obstructed for a long period of time. Such delay in the resumption of the synthesis of cholic acid was noted previously by Ravdin *et al* (1), and a similar delay in synthesis of "bile acids" was observed in some of the more severely obstructed cases of Greene *et al* (18, 19) by application of an analytical method of somewhat questionable specificity. In the cases studied in the present in-

[4] See Case Histories.

vestigation all of the various bile acids were entirely or nearly lacking from the bile excreted during the first few days following the operation. However, the excretion of desoxycholic acid and the total dihydroxycholanic acid fraction (which includes desoxycholic acid) was resumed somewhat earlier than the excretion of cholic acid. By means of other analytical methods, Breusch and Johnston (20) obtained evidence for a similar effect. The more rapid appearance of the dihydroxycholanic acids was particularly striking in the case of A. M. (Table IV), but was less marked in the cases of C. D. and C. M. (Tables I and VI). In all cases in which both the total dihydroxycholanic acid fraction and desoxycholic acid, a specific member of that fraction, were determined, the quantity of total dihydroxycholanic acids exceeded the amount of desoxycholic acid. This is evidence for the presence of one or more additional members of the dihydroxycholanic acid series. Inasmuch as Wieland *et al* (9, 21) have isolated chenodesoxycholic acid,[5] a dihydroxycholanic acid, from gallbladder bile collected from cadavers, it is likely that the differences between our figures for total dihydroxycholanic acids and the corresponding figures for desoxycholic acid are a measure of the amounts of chenodesoxycholic acid present. However, as mentioned below, in order to obtain values for chenodesoxycholic acid in those cases in which hog bile was administered to the patients it would be necessary also to subtract the figure for hyodesoxycholic acid since this bile acid is included in the dihydroxycholanic acid fraction. The traces of "hyodesoxycholic acid" recorded in Table IV for periods in which hog bile was not administered to this patient correspond to "blank" values obtained with the polarimetric method for the determination of this bile acid and probably do not represent the presence of true hyodesoxycholic acid in these samples. Average figures for these "blanks" were used in correcting all values obtained for hyodesoxycholic acid by this method.

The biliary excretion after approximately the twentieth day following the operation probably represents an *approach* to normal hepatic bile, at least in a few of the cases in which there appeared to be minimal liver damage from obstruction and cholangitis. The extent of such damage is difficult to assess, but, of the cases studied in greatest detail, it would appear that C. D. (Tables I and II) most nearly attained a normal state of biliary

TABLE I

Excretion of various bile acids in the choledochostomy drainage bile of patient C. D., and the effect of oral administration of desiccated hog bile

Days post-operative	Hyodesoxycholic acid content of hog bile administered	Average volume of bile excreted per day	Concentration of bile acids in drainage bile				
			Cholic acid	Desoxycholic acid	Hyodesoxycholic acid	Total dihydroxycholanic acid	Total bile acids
	millimoles per day	*ml.*	*millimoles per liter*				
2	0	425	0	0		0	0
3–4	0	370	0	trace		trace	trace
5–6	0	460	0	0.10		0.10	0.10
7–8	0	540	trace	0.15		0.20	0.20
9–10	0	380	0.3	0.20		0.32	0.62
11	0	510	0.6	0.40		0.63	1.23
12	0	580	1.2	0.46		0.82	2.02
13	0	540	2.9	0.70		1.2	4.1
14	0	530	4.1	0.95		1.7	5.8
15	0	610	5.8	1.6		2.4	8.2
16	0	505	6.9	2.1		3.6	10.5
17	0	605	8.3	3.0		5.2	13.5
18	0	550	9.4	3.0		5.4	14.8
19	0	560	9.8	3.4		5.7	15.5
20	0	590	9.2	3.1		5.4	14.6
21	0	600	10.0	3.2		5.8	15.8
22	2.8	985	10.2	3.0	2.5	8.1	18.3
23	2.8	1,010	9.7	3.3	2.4	7.7	17.4
24	0	790	12.2	3.9		6.5	18.7
25	0	685	14.3	4.6		8.3	22.6

excretion. Even in this case it is doubtful whether the maximum *concentration* of the bile acids had been attained at the termination of the period of drainage inasmuch as the concentrations were continuing to increase at that time. However, the *relative proportions* of the various bile acids were rather constant after the twentieth day. The average proportion of cholic acid to desoxycholic acid to chenodesoxycholic acid for days 20, 21, 24, and 25 was 3:0.98:0.74. Days 22 and 23 were omitted from the calculation because hog bile was ad-

[5] Wieland isolated this bile acid from the bile of man in 1924, and he gave it the name, anthropodesoxycholic acid. Windaus in the same year announced the isolation of the identical compound from the bile of the goose and named it chenodesoxycholic acid. No general agreement regarding the name appears to have been reached, but the name proposed by Windaus seems to be favored since the prefix, *cheno*, was used first by Heintz and Wislicenus in connection with an imperfectly characterized acid which they isolated from bile of the goose in 1859.

TABLE II

The excretion of conjugated bile acids in the choledochostomy drainage bile of patient C. D., and the effect of oral administration of desiccated hog bile

Days post-operative	Hyodesoxycholic acid content of hog bile administered	Concentration of bile acids in drainage bile			
		Total bile acids	Total conjugated bile acids	Tauro-conjugated bile acids	Glyco-conjugated bile acids
	millimoles per day	*millimoles per liter*			
2	0	0	0		
3–4	0	trace	trace		
5–6	0	0.10	0.06	0.02	0.04
7–8	0	0.20	0.13	0.05	0.08
9–10	0	0.62	0.45	0.18	0.27
11	0	1.23	0.92	0.38	0.54
12	0	2.02	1.55	0.61	0.94
13	0	4.1	3.3	1.35	1.95
14	0	5.8	4.8	2.1	2.7
15	0	8.2	6.4	2.6	3.8
16	0	10.5	8.5	3.8	4.7
17	0	13.5	11.5	5.2	6.3
18	0	14.8	12.7	5.9	6.8
19	0	15.5	13.5	5.8	7.7
20	0	14.6	13.0	5.9	7.1
21	0	15.8	13.3	5.8	7.5
22	2.8	18.3	15.9	5.6	10.3
23	2.8	17.4	14.9	5.2	9.7
24	0	18.7	15.1	6.8	8.3
25	0	22.6	19.2	8.8	10.4

ministered on those days. The *proportions* of these 3 bile acids in hepatic bile of this case are in good agreement with those obtained by Wieland *et al* (9, 21) by calculation from the isolation of bile acids from pooled samples of gallbladder bile obtained from human cadavers. However, the absolute concentrations of these bile acids obviously were smaller in hepatic bile than in gallbladder bile. On the other hand, these data for the relative proportions of the bile acids do not agree with data reported by Colp and Doubilet (22) for the ratios of cholic acid to "desoxycholic acid" in gallbladder bile of a series of cases. However, these authors stated that the method which they used for determination of "desoxycholic acid" was not specific for that acid and probably included chenodesoxycholic acid, lithocholic acid, and possibly other bile acids of unknown nature.

A considerable fraction of the bile acids excreted by C. D. (Table II) was unconjugated during the first 10 days following the operation, but the percentage of the total bile acids in the conjugated form rose to 85 during the latter part of the period of drainage. The average ratio of glyco-conjugated bile acids to tauro-conjugated acids was 1.3: 1 in the bile of C. D. during periods when hog bile was not administered.

In the case of C. M. (Table VI) with apparently greater liver damage than C. D., recovery of the synthesis of bile acids was delayed longer, and the bile acid concentrations attained after 40 days were approximately ½ those which occurred in the bile of C. D. after 20 days. During the first 3 weeks following the operation, the bile excreted by patient C. M. contained much sediment, and it was pale brown in color and possessed an offensive odor. Thereafter, there was progressive improvement in the appearance of the bile, and after approximately the thirty-fourth day, the bile was fairly clear and of normal appearance. This improvement in the appearance of the bile was accompanied by a rapid increase in the excretion of bile acids. Excretion of desoxycholic acid and other dihydroxycholanic acids (presumably chenodesoxycholic acid) by C. M. was resumed before the excretion of cholic acid. However, on days 39 and 40 the *proportion* of cholic acid to desoxycholic acid to chenodesoxycholic acid was 3:1:0.7 which is in good agreement with the proportion found in the case of C. D. As late as the fourteenth and eighteenth days following the operation, 56 and 49 per cent of the total bile acids were unconjugated, but the percentage of unconjugated acids had decreased to an average of approximately 11 at the end of the period of drainage (calculated from the data for days 39 and 40, during which ox bile was not administered). At this time the average glyco/tauro ratio was 1.91: 1 (Table VII).

Patient A. M. (Tables IV and V) was in rather

TABLE III

The enterohepatic circulation of glycohyodesoxycholic acid; patient C. D.

Glycohyodesoxycholic acid content of dried hog bile administered	Duration of enterohepatic circulation	Glycohyodesoxycholic acid content of bile excreted at end of period of circulation	Loss of glycohyodesoxycholic acid during circulation
millimoles	*hours*	*millimoles*	*percentage of initial amount*
2.6	24	1.9	27
4.75	24	2.9	39
10.0	24	6.3	37
2.6	48	1.0	61.5
4.75	48	1.4	70.5
4.75	96	0.5	89.5

TABLE IV

Excretion of various bile acids in the choledochostomy drainage bile of patient A. M., and the effect of oral administration of desiccated hog bile

Days post-operative	Hyodesoxycholic acid content of hog bile administered	Average volume of bile excreted per day	Concentration of bile acids in drainage bile			
			Cholic acid	Hyodesoxycholic acid	Total dihydroxycholanic acids	Total bile acids
	millimoles per day	*ml.*	*millimoles per liter*			
2	0	405	0	0	trace	trace
3	0	450	0	0	0.45	0.45
4	0	510	0	0	0.62	0.62
6	0	500	0	0	1.45	1.45
8	0	545	0	0.1	2.85	2.85
10	0	630	trace	0.1	3.92	3.92
11	0	710	trace	0.1	5.10	5.1
12–13	0	790	1.47	0.19	4.72	6.2
14–16	0	850	2.94	0.21	4.26	7.2
17	0	730 (?)	3.69	0.08	2.50	6.2
18	0	810	5.05	0.12	3.82	8.9
19	0	884	8.12	0.13	4.06	12.2
20–22	7.34	1,030	10.8	5.1	8.70	19.5
23–26	7.34	1,120	10.8	5.6	9.14	19.9
27–28	7.34	1,140	12.7	5.2	8.72	21.4
29	0	920	15.1	0.22	5.87	21.0
30	0	895	16.5	0.20	7.04	23.5

TABLE V

The excretion of conjugated bile acids in the choledochostomy drainage bile of patient A. M., and the effect of oral administration of desiccated hog bile

Days post-operative	Hyodesoxycholic acid content of hog bile administered	Concentration of bile acids in drainage bile			
		Total bile acids	Total conjugated bile acids	Tauro-conjugated bile acids	Glyco-conjugated bile acids
	millimoles per day	*millimoles per liter*			
2	0	trace	0		
3	0	0.45	trace		
4	0	0.62	0.25	0.12	0.13
6	0	1.45	0.79	0.31	0.48
8	0	2.85	1.95	0.70	1.25
10	0	3.92	3.42	1.24	2.18
11	0	5.1	5.0	2.10	2.90
12–13	0	6.2	6.03	3.30	2.73
14–16	0	7.2	6.98	3.21	3.77
17	0	6.2	6.10	2.80	3.30
18	0	8.9	8.80	4.23	4.57
19	0	12.2	11.8	5.66	6.14
20–22	7.34	19.5	19.6	7.40	12.2
23–26	7.34	19.9	19.8	7.60	12.2
27–28	7.34	21.4	21.2	8.60	12.6
29	0	21.0	20.1	9.4	10.7
30	0	23.5	21.9	10.3	11.6

poor condition. Nevertheless, the excretion of cholic acid was resumed almost as soon after operation as occurred in the case of C. D., and the recovery of the excretion of dihydroxycholanic acids actually was more rapid than in the case of C. D. In fact, it was more rapid than occurred in any of the other cases which we investigated. Unfortunately, analyses for desoxycholic acid were not conducted on samples of bile from A. M., therefore the nature of the dihydroxycholanic acids was not determined. Again a large fraction of the total bile acids was unconjugated during the first six days after the operation, but thereafter recovery of the ability to conjugate the bile acids was rapid.

(2) *Excretion of bile acids in drainage bile following oral administration of desiccated bile:* Following oral administration of dried gallbladder bile of the hog,[6] excretion of hyodesoxycholic acid, the predominant bile acid of hog bile, in the choledochostomy drainage bile, commenced after 1 to 2 hours. Thereafter, the excretion rose to a peak at about 7 to 10 hours, then gradually declined, the total period of excretion comprising 18 to 20 hours.[7] In Table I (case C. D.) the concentration of hyodesoxycholic acid in the bile collected during a 24-hour period following administration of hog bile is recorded, together with the total volume of bile. From these data the total *amounts* of hyodesoxycholic acid excreted were calculated. The average excretion of hyodesoxycholic acid by this patient was 87.5 per cent of the amount admin-

[6] Preparations of desiccated gallbladder bile of the hog (trade name, *Desicol*) and the ox were supplied by Parke, Davis and Company.

[7] This period of circulation or total "clearance-time" is considerably longer than that reported for man by Josephson (23), but it is comparable with the data for hogs (12) and for dogs (24, 25). Josephson calculated the clearance-time by observing the time required for biliary excretion of the natural cholates to return to the normal secretory level from the high level induced by recirculation of bile or by administration of cholates. Inasmuch as the normal rate of excretion of cholates is subject to some variation, it would be difficult to determine the total clearance-time precisely by that method. On the other hand, the time required for attainment of the *peak* of clearance of hyodesoxycholate in our experiments was comparable with the total clearance-time reported by Josephson. It is probable that enterohepatic circulation may have been somewhat delayed in the patients of our series as compared with the relatively normal subjects studied by Josephson. In addition, the circulation of hyodesoxycholate may require a longer time than the circulation of the natural cholates.

istered. In the case of A. M. (Table IV), hog bile was administered daily, as recorded in column 2, from the twentieth to the twenty-eighth day. The drainage bile was pooled for the intervals shown in column 1, and the *average* daily volume and *average* daily excretion were calculated. The average excretion of hyodesoxycholic acid by this patient was 78.5 per cent of the amount administered. In Tables II and V it can be seen that in the 24-hour period following administration of hog bile there was an increase in the ratio of glyco-conjugated acids to tauro-conjugated. Inasmuch as hyodesoxycholic acid of hog bile is conjugated solely with glycine, this increase in the glyco/tauro ratio of the drainage bile, together with the excretion of hyodesoxycholic acid, suggests strongly that glycohyodesoxycholic acid was absorbed from the intestine and excreted unchanged in the drainage bile. The efficiency of utilization of this foreign bile acid by these patients appears to be somewhat less than the efficiency with which glycocholic acid was handled by hogs with biliary fistulae (12). However, it is doubtful whether the bile collected by drainage through a T-tube represents the *entire* biliary secretion even under carefully supervised conditions.

TABLE VI

Excretion of various bile acids in the choledochostomy drainage bile of patient C. M., and the effect of oral administration of desiccated ox bile

Days post-operative	Cholic acid content of ox bile administered	Volume of bile excreted per day	Concentration of bile acids in drainage bile			
			Cholic acid	Desoxycholic acid	Total dihydroxycholanic acids	Total bile acids
	millimoles per day	*ml.*	*millimoles per liter*			
2	0	615	0	0.08	0.12	0.12
10	0	705	0	0.11	0.19	0.19
14	0	640	trace	0.32	0.55	0.55
18	0	620	0.1	0.37	0.69	0.79
19	1.2	990	0.9	0.48	0.81	1.71
21	1.2	960	1.2	0.51	0.85	2.05
22	1.2	1,005	1.4	0.39	0.80	2.20
24	1.2	1,100	1.3	0.56	0.92	2.22
25	1.2	910	1.8	0.49	0.90	2.70
26	1.2	940	2.4	0.65	1.10	3.50
28	1.2	915	3.1	0.78	1.35	4.45
30	1.2	898	4.3	1.12	2.04	6.34
32	1.2	930	5.2	1.43	2.58	7.78
34	1.2	905	5.9	1.74	3.11	9.01
36	1.2	900	6.3	1.82	3.23	9.53
38	1.2	895	6.6	1.81	3.10	9.70
39	0	905	5.7	1.91	3.29	8.99
40	0	890	5.9	2.02	3.36	9.26

It was of considerable interest to study the enterohepatic circulation of bile acids in these patients. The hyodesoxycholic acid of hog bile provided a "tracer" for study of such circulation since this bile acid is foreign to the normal biliary secretion of man. These studies were conducted as described in our paper on enterohepatic circulation in hogs (12). Following oral administration of hog bile containing the quantity of glycohyodesoxycholic acid recorded in column 1 of Table III, the external choledochostomy drainage tube was clamped shut in order to direct the flow of bile into the intestine. Following the period of circulation recorded in column 2, the clamp was removed and the bile was collected for a 20-hour period for determination of the amount of hyodesoxycholic acid remaining at that time (column 3). The percentage loss of glycohyodesoxycholic acid is recorded in column 4. Even after 4 days, some of the hyodesoxycholic acid remained in the circuit. Again the efficiency of circulation was somewhat less than that of the hog, but, nevertheless, such circulation provides an important mechanism for the conservation of bile acids.

Inasmuch as the bile acid composition of ox bile is very similar to that of man, it was more difficult to assess quantitatively the effect of administration of ox bile to these patients. However, it is appar-

TABLE VII

The excretion of conjugated bile acids in the choledochostomy drainage bile of patient C. M., and the effect of oral administration of desiccated ox bile

Days post-operative	Cholic acid content of ox bile administered	Concentration of bile acids in drainage bile			
		Total bile acids	Total conjugated bile acids	Tauro-conjugated bile acids	Glyco-conjugated bile acids
	millimoles per day	*millimoles per liter*			
2	0	0.12	trace		
10	0	0.19	trace		
14	0	0.55	0.24	0.19	0.05
18	0	0.79	0.40	0.20	0.20
19	1.2	1.71	1.27	0.55	0.72
21	1.2	2.05	1.56	0.66	0.90
22	1.2	2.20	1.89	0.72	1.17
24	1.2	2.22	1.97	0.70	1.27
25	1.2	2.70	2.20	0.81	1.39
26	1.2	3.50	2.85	0.99	1.86
28	1.2	4.45	3.52	1.39	2.13
30	1.2	6.34	5.64	1.89	3.75
32	1.2	7.78	7.05	2.42	4.63
34	1.2	9.01	8.02	2.73	5.29
36	1.2	9.53	8.64	2.91	5.73
38	1.2	9.70	8.83	3.12	5.71
39	0	8.99	7.87	2.65	5.22
40	0	9.26	8.30	2.91	5.39

TABLE VIII

Excretion of cholic acid in the choledochostomy drainage bile of patient F. W., and the effect of oral administration of desiccated ox bile

Days post-operative	Cholic acid content of ox bile administered	Volume of bile excreted per day	Concentration of cholic acid in drainage bile
	millimoles per day	*ml.*	*millimoles per liter*
2	0	690	0
3	0	520	0
4	0	580	0
5	0	625	trace
6	0	680	trace
7	0	610	0.12
8	1.42	950	1.23
9	1.42	1,025	1.39
10	1.42	1,190	1.24
11	1.42	1,080	1.47
12	1.42	1,140	1.66

ent from Tables VI and VIII that administration of desiccated ox bile to these patients, in the early post-operative period before recovery of the excretion of bile acids, was followed by an immediate increase in the excretion of bile acids, particularly cholic acid. Inasmuch as the average ratio of cholic acid to desoxycholic acid is 5.5:1 in ox bile (10) as compared to 3:1 in the bile of man, it is to be expected that the administration of ox bile to human patients would cause a proportionately greater increase in cholic acid. Administration of ox bile did not produce a significant change in the glyco/tauro ratio in the bile of C. M. (Table VII) if the period from days 30 to 38, during which ox bile was administered, is compared with days 39 and 40, during which administration of ox bile was discontinued. This is to be expected in view of the similarity in the conjugation of bile acids of man and the ox.

Administration of either hog bile or ox bile to these patients produced an increase in the *volume* of bile excreted during the ensuing 24 hours. The question of the effect of the administration of animal bile upon the *synthesis* of bile acids by the patient is important but is difficult to decide from our data inasmuch as the synthesis of bile acids was increasing progressively in most of the cases *before* animal bile was administered. The *quantity* of bile acids excreted per day can be calculated from the corresponding data for the *volume* of bile and the *concentrations* of the bile acids. Such data for the quantity of bile acids excreted in the bile per day are recorded for three of the cases in Figures 1 to 3. Also, in these figures data for bile

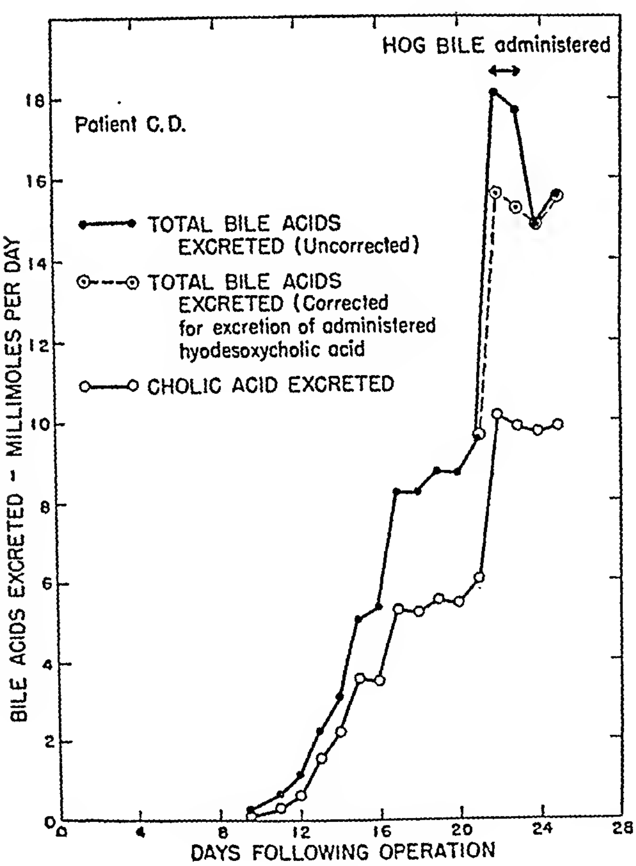

FIG. 1

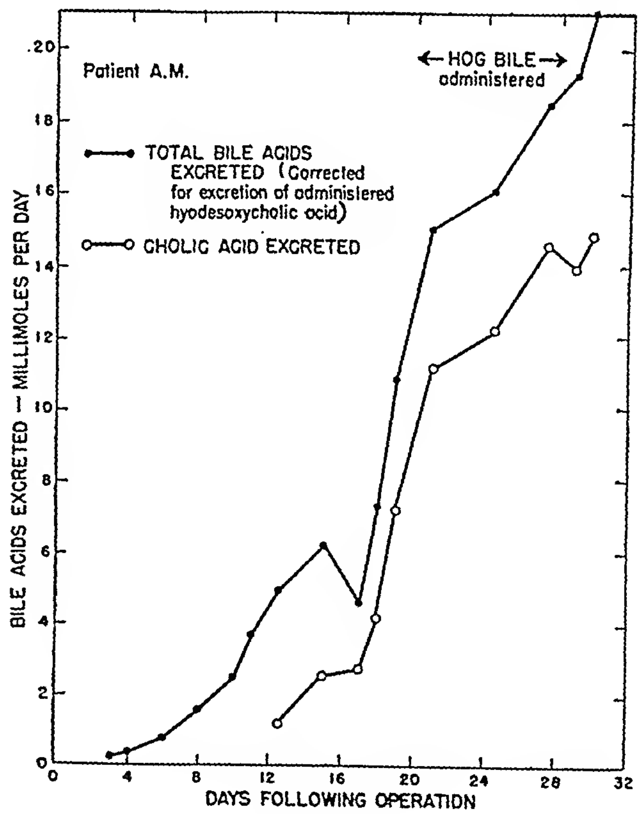

FIG. 2

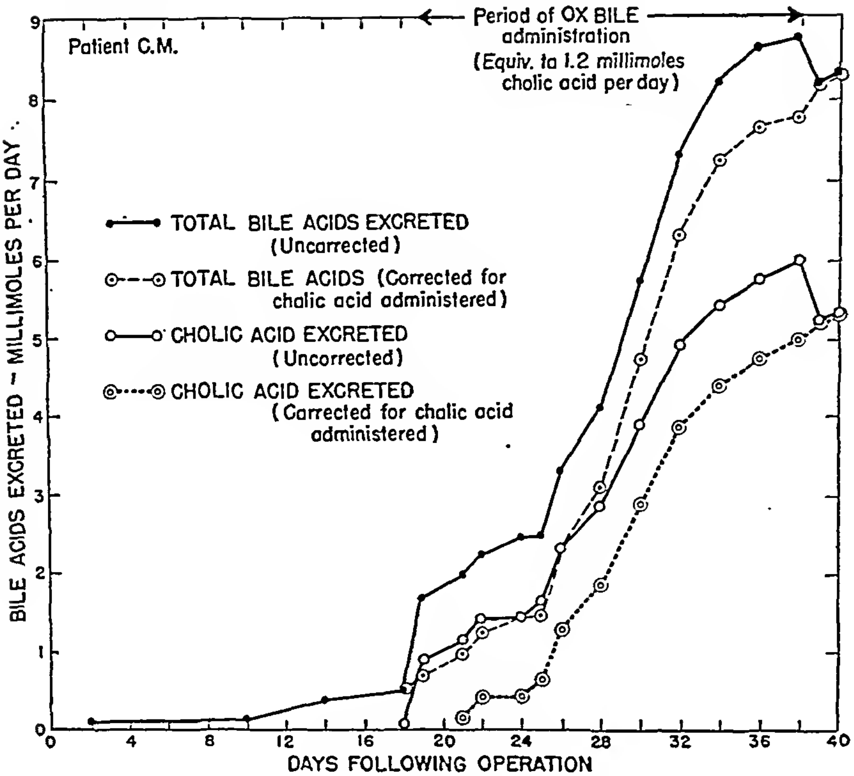

FIG. 3

acid excretion are presented which have been "corrected" for the excretion of the bile acids contained in the administered animal bile, thus yielding data for the production of bile acids by the patient. It can be observed from these corrected curves that administration of bile of the ox or the hog did not depress the production of bile acids by the patients although there was a slight leveling of the slope of the curves in the case of A. M. (Figure 2) who received the largest doses of hog bile. On the other hand, there *appears* to have been some stimulation of bile acid production in the cases of C. D. (Figure 1) and C. M. (Figure 3). However, such a conclusion would seem to be unwarranted from the limited data available in view of the difficulties mentioned above. In the case of C. D. (Figure 1), the sharp increase in excretion of bile acids upon administration of hog bile might have been due to release of a slight obstruction to the flow of bile as a result of the pressure of the increased bile flow caused by the administered hog bile. However, the increase in *concentration* of the bile acids, in addition to the increase in *volume* of bile excreted, following administration of hog bile (Table I), is somewhat suggestive of a stimulatory effect. Nevertheless, if such an effect occurred, it must be assumed that it persisted *after cessation* of hog bile administration in view of the continued increase in the *concentration* of the bile acids (Table I) and of the maintenance of the *quantity* of bile acids excreted (Figure 1). In the case of C. M. (Tables VI and VII and Figure 3) there was progressive increase in the excretion of bile acids throughout the period of ox bile administration, and the production of bile acids by the patient was maintained during the 2 days of observation following cessation of ox bile administration (corrected curves of Figures 3). Again, stimulation of bile acid production cannot be claimed since similar progressive increases in bile acid production have been observed in other patients during periods in which ox bile was not administered. During the period of observation this patient did not attain so high a level of bile acid excretion as did patients C. D. and A. M., but, as mentioned previously, the bile of this patient was quite abnormal during the first 3 weeks (improving slowly thereafter) suggesting

a pathological state of the liver or bile ducts, or both.

While the possible stimulation of bile acid production by administration of animal bile to these patients cannot be decided conclusively from these data, it seems desirable to emphasize again those effects which are demonstrated unequivocally, *i.e.*, the increase in *volume* of bile-flow and the absorption and reexcretion of the bile acids supplied in the animal bile. The intestinal absorption of these "foreign" bile acids by the patients suggests that these animal biles can be used successfully in replacement therapy. Additional evidence of their successful function in replacing the bile of the patient has been provided by observations of increases in the values for prothrombin assays during periods when animal bile was administered.

In previous papers (12, 13) we have demonstrated that normally the concentration of cholates in blood of the peripheral circulation is less than 1 mgm. per 100 ml., but appreciably greater concentrations of cholates appear in peripheral blood of men and dogs with biliary obstruction (13, 23) except in those cases in which the obstruction is of such duration that the damaged liver has lost the ability to synthesize cholates. However, even in the latter condition, the oral administration of cholates to animals with biliary obstruction is followed by the appearance of cholates in the circulation (12, 13). In Table IX there are recorded the results of analyses for cholates in blood samples obtained from a patient with biliary obstruction.

TABLE IX

Concentrations of cholates in blood of the peripheral circulation of patient C. S. before and after operation for the removal of obstruction of the bile duct, and the effect of oral administration of hog and ox biles

Pre-operative period	Desiccated bile administered orally	Cholate concentration in whole blood, "cholic acid"
days	*grams per day*	*mgm. per 100 ml.*
36–18	1.7 (hog)	
32		2.65
21		2.32
18		1.24
13–6	1.0 (ox)	
8		7.69
At operation		1.91
Post-operative period		
days		
7–20	1.0 (ox)	
10		0.14
18		0.09
31	9.0 (ox)	
32	0.0	0.80
40	0.0	0

This patient (C. S.), a 45-yr.-old black male, had had a cholecystectomy performed 6 months previous to admission, and an attempted drainage of his common duct one month after the cholecystectomy because of increasing jaundice. Following his operation a small amount of bile drained from his wound but his jaundice did not disappear. At the time of admission his skin had been markedly icteric for 6 months, he had had frequent intermittent attacks of upper abdominal pain associated with chills and fever, and suffered intense itching. His stools had been persistently acholic. Shortly after admission the Van den Bergh was direct, immediate with 9.3 mgm. bilirubin per 100 ml. of blood.

He was operated upon 37 days after admission and no evidence of the common duct was found. There was a small scarred area in the hilus of the liver from which thin, pale green bile was aspirated. The duodenum was sufficiently mobilized to permit direct anastomosis, over a pezzar catheter, with the hepatic duct, the catheter being brought out to the anterior abdominal wall through the stomach. Drainage of bile through the catheter was continued for 6 weeks. The catheter was then removed, and within 24 hours drainage from the wound had stopped.

Samples were obtained during pre- and post-operative periods and during the administration of desiccated bile of the hog and the ox. During the thirty-sixth to the eighteenth day before the operation there was a decline in the concentration of cholates in the peripheral blood which probably was indicative of a decline in the ability of the liver to synthesize cholates. This decline in the concentration of cholates occurred during a period when desiccated hog bile was being administered orally. However, hog bile does not contain cholates (8, 12), and our method (13) was specific for the determination of that bile salt, either unconjugated or conjugated with glycine or taurine. At the time of these studies, we did not have available a method for the determination of hyodesoxycholates in blood; therefore, we were un-

able to study the interesting question of the possible appearance of that bile salt of the hog in the peripheral circulation of obstructed patients receiving hog bile by mouth. On the eighteenth day (pre-operative), administration of hog bile was discontinued. During the period from the thirteenth to the sixth pre-operative day, a daily oral dose of 1 gram of desiccated ox bile (which contains cholates) was administered. The concentration of cholate in the peripheral blood had risen to 7.69 mgm. per 100 ml. by the eighth pre-operative day. Administration of ox bile was discontinued on the sixth day before operation, and at the time of operation the concentration of cholate had declined again to 1.91 mgm. per 100 ml. of blood. After removal of the obstruction by operation, the concentration of cholate in the peripheral circulation remained low even when large doses of dried ox bile were administered orally.

SUMMARY

Bile samples collected post-operatively by choledochostomy drainage from a series of 11 cases were analyzed for cholic, desoxycholic, and total dihydroxycholanic acids and for glyco, tauro, and total conjugated bile acids. A period of 10 to 20 days after operation usually elapsed before resumption of synthesis of these bile acids by patients whose biliary tracts had been severely obstructed. In some of the cases, the excretion of desoxycholic acid and the total dihydroxycholanic acid fraction was resumed somewhat earlier than the excretion of cholic acid. In most cases, a considerable fraction of the bile acids was unconjugated during the first week or two following the operation, but usually recovery of the ability to conjugate the bile acids (with glycine or taurine) did not lag far behind recovery of the synthesis of the steroid nucleus of the bile acids.

After a recovery period of 20 to 25 days, the average proportion of cholic acid to desoxycholic acid to chenodesoxycholic acid was 3:1:0.75 in the bile of a patient with apparently minimal liver damage. Similar proportions between these bile acids were obtained for most of the other cases during the late post-operative period, but the absolute *concentrations* of the bile acids were smaller in the bile of those patients with the greatest liver damage.

Oral administration of desiccated gallbladder bile of the hog to these patients was followed by the biliary excretion of hyodesoxycholic acid, a bile acid which normally is foreign to the bile of man but which is a principal component of hog bile in which it is found conjugated with glycine. There also was an increase in the glyco/tauro coefficient which suggests that glycohyodesoxycholic acid was absorbed and reexcreted unchanged in the drainage bile. This foreign bile acid was handled fairly efficiently in the enterohepatic circulation, a portion remaining in the circuit even after 4 days.

Following oral administration of desiccated gallbladder bile of the ox to these patients, there were increases in the excretion of cholic and desoxycholic acids which were proportional to the amounts of these bile acids in the administered ox bile.

Preparations of desiccated bile of the hog and the ox provide a convenient and effective means for replacement of bile during choledochostomy drainage. Both preparations are well tolerated, both stimulate an increase in the volume of bile-flow, and both provide bile acids which are effective in supplementing the output of bile acids by the patient, apparently without inhibiting the synthesis of bile acids.

Cholates normally are present in blood of the peripheral circulation in concentrations less than 1 mgm. per 100 ml., but the concentrations increase appreciably when the biliary tract is obstructed unless the synthesis of cholates is decreased by liver damage. Even in the latter case, the oral administration of cholates, or ox bile containing cholates, produces an increase in the concentration of cholates in the peripheral circulation in contrast to the absence of this effect when the biliary tract is not obstructed.

CASE HISTORIES

Case 1.—F. W., a 53-year-old white male, entered the hospital 5-11-40 because of fever, chills of 5 days' duration, and epigastric pain for 3 days. He had noticed a yellow tinge to his skin for several days. More than a year previous to this time he had stopped eating pork; over this same period he had suffered heart burn regularly. He had lost 25 pounds in the past year.

Temperature 100.8°, pulse 100, respiration 20, blood pressure 106/60. Icterus was obvious. There was rigidity in the right upper quadrant with tenderness in this area. The liver was 2 cm. below the costal margin.

There was a right inguinal hernia present. There was stasis dermatitis on both legs.

Van den Bergh 1.3 mgm., direct; blood urea nitrogen 143; serum albumin 4.6 per cent, globulin 4.0 per cent. Urine showed only faint trace of albumin consistently. Hb 11.5 grams, RBC 2,240,000, WBC 6,400, polys 85 per cent. Opaque shadows in flat film suggested gallstones. The patient was treated symptomatically, given blood transfusions and fluid by vein. Despite the high, maintained blood urea the patient appeared moderately improved, and it was believed that the high urea was associated with liver disease.

Operation was performed on 5-21-40, at which time a lobulated scarred liver was found. Marked adhesions were present about the gallbladder and common duct. The gallbladder was exposed with some difficulty. It contained numerous small stones. Despite the need for common duct exploration, on account of the difficulties encountered, it was decided to drain the common duct through the dilated cystic duct. The proximal portion of the gallbladder and the cystic duct were sutured about the tube for future use if needed. Bile readily drained from the tube. The patient was given several transfusions of whole blood post-operatively. His blood pressure quickly becoming stabilized at 110/60. Within five days the temperature returned to normal, the highest post-operative temperature being 102°. Despite the generally improved appearance of the patient, he complained of feeling weak and ate very little. Within 2 weeks post-operatively it became more difficult to get him to take nourishment by mouth. The tube was removed 21 days after operation. The patient expired on 6-13-40. Postmortem examination was not performed.

Case 2. C. M., a 50-year-old colored housewife entered the hospital 10-26-40, because of acute epigastric pain of 10 hours' duration. The pain radiated to the interscapular area and was associated with nausea and vomiting. For 15 years she had had intolerance to fatty foods. On admission she was restless, moving about with recurrent attacks of acute pain. There was rigidity of the right upper quadrant. Temperature 102°, pulse 100, respiration 24, blood pressure 90/60.

Shortly after admission following administration of morphine sulphate, nitroglycerine and intravenous fluids, the blood pressure was 150/104 and the patient became more comfortable. Hb 11.5 grams, WBC 7,300, urinalysis showed faint trace of albumin. Van den Bergh direct, immediate, 1.9 mgm per 100 ml. All evidence of icterus gone, icteric index being 7.5.; operation was performed on 11-9-40 at which time a chronically inflamed gallbladder was removed and the dilated thickened common duct was explored. A T-tube was placed in the common duct for drainage. The capsule of the liver was thickened and there was increased trabeculation of the liver. No obstruction was found. Following operation, the patient's blood pressure dropped to 90/50, but following plasma infusion, rose to 140/100. After a few stormy days, her temperature, pulse, respiration, and blood pressure became stabilized at normal levels. Drainage was carried on for 40 days. The patient was discharged to the outpatient department.

Case 3. C. D., a 58-year-old white woman, was admitted to the medical service because of severe epigastric pain of 1 day's duration. This was but one of many repeated similar attacks which she had had for the preceeding 7 years. These attacks usually began as pain in the epigastrium or in the right upper quadrant. There had been no jaundice noted, but there had been chills and fever on occasion, and she had noted dark urine. Temperature 98.4°, pulse 98, respiration 20, blood pressure 130/70, weight 88½ pounds.

On physical examination there were definite tenderness and slight rigidity of the right upper quadrant. The right kidney was palpable and there was tenderness in the right costovertebral angle. The liver was palpable 2 centimeters below the costal margin. Other positive findings were a right inguinal hernia and external hemorrhoids. Icterus was not obvious clinically. The Kline test was negative, hemoglobin 11 grams RBC 4,340,000, WBC 11,800, polys 86 per cent. Serum albumin 3.5 per cent, globulin 1.6 per cent, van den Bergh 0.6 mgm. per 100 cc. Flat film of the abdomen revealed 2 large radiopaque shadows which were interpreted as gallstones. Barium study revealed diverticula of the duodenum. Intravenous pyelogram revealed nothing unusual.

Immediately following admission, pain and tenderness subsided, but 11 days later she had pain in the epigastrium, vomiting, and return of tenderness associated with chills and a fever of 103°. This promptly subsided to recur 2 days later. Similar but milder attacks occurred every 2 to 4 days thereafter until she was operated upon 1-8-40. At operation a contracted thickened gallbladder was found with a 1½ cm. stone imbedded in the fundus. The common duct measured more than 2 cm. in diameter and was filled with a large, putty-like mass, greenish brown in color, which formed a cast of the common duct. This material was removed, the duct washed clear, and a large T-tube placed in the common duct. The gallbladder was removed. The liver was found to be large and extended 4 cm. below the costal rim. The surface was finely granular and green tinted.

Following operation the patient's condition was satisfactory. Drainage was continued for 25 days, following which, after determining patency of the duct, the tube was removed. The patient returned a year later for repair of her inguinal hernia at which time she had had no recurrence of her symptoms and was able to eat without restriction.

Case 4. A. M., a 61-year-old Turkish male, was admitted to the medical service on 10-23-38 because of right upper quadrant pain, jaundice, and vomiting. For 2 years previously he had had intermittent attacks of right upper quadrant pain with nausea and vomiting. He had lost seventy pounds in the 2 years, but stated he ate very little for fear of precipitating an attack. Two days previously he had had an attack which was more severe than usual which was accompanied with chills and fever, and he noted for the first time that he was jaundiced. Upon

examination he was found to be markedly icteric and the skin and mucus membranes were quite dry. Temperature 99°, pulse 100, respiration 22, blood pressure 100/70. Moist rales were present over the right base posteriorly. The liver edge could be felt 3 cm. below the costal margin in the midclavicular line and there were marked tenderness and moderate rigidity of the right upper quadrant. The spleen was palpable on deep inspiration. There was pitting edema of dependent parts. On admission, the urine contained bile, albumin 4 and an occasional white cell. Hb 12.5 grams, RBC 4,300,000, WBC 31,300, polys 96 per cent. Kline test negative, van den Bergh immediate, direct, 4.5 mgm. There was a trace of urobilinogen in the urine, blood cholesterol 228 mgm. On 11-14-38, three weeks after admission, his white count was 8,800 and his hemoglobin 8.0 grams, serum albumin 3.3, globulin 2.8, clotting time 3½ minutes, bleeding time 4 minutes, 10 seconds, prothrombin 52 per cent.

The patient was treated symptomatically. He was given 0.4 gram pig bile t.i.d. with several experimental preparations of vitamin K given orally.

Just preceding operation, which was performed on 1-17-39, the prothrombin was 58 per cent, hemoglobin 12.6 grams, WBC 7,800, van den Bergh 0.75 mgm. At operation the liver appeared but slightly enlarged, was paler than normal, and the capsule was moderately thickened. The gallbladder contained numerous stones and was removed. The common duct was thickened and admitted the index finger easily. Three stones were removed from the duct, following which the probe passed easily into the duodenum and into the liver. A T-tube was sutured into the common duct and brought out through the abdominal wall. Following operation he had no unusual difficulties and was discharged in good condition.

BIBLIOGRAPHY

1. Ravdin, I. S., Johnston, C. G., Riegel, C., and Wright, S. L., A study of human liver bile after release of common duct obstruction. J. Clin. Invest., 1933, 12, 659.
2. Ravdin, I. S., Riegel, C., Johnston, C. G., and Morrison, P. J., Studies in biliary tract disease. J. A. M. A., 1934, 103, 1504.
3. Irvin, J. L., Merker, H., Anderson, C. E., and Johnston, C. G., The comparison of desiccated and normal hog gallbladder bile. J. Biol. Chem., 1939, 131, 439.
4. Johnston, C. G., Preoperative and postoperative treatment in cases of obstructive jaundice. Surgery, 1938, 3, 875.
5. Winfield, J. M., The use of dried bile as a therapeutic agent. J. Michigan M. Soc., 1938, 37, 798.
6. Sobotka, H., Physiological Chemistry of the Bile. Williams and Wilkins, Baltimore, 1937.
7. Schoenheimer, R., Andrews, E., and Hrdina, L., Uber das Auftreten ungekuppelter Gallensauren in menschlicher Galle. Ztschr. f. physiol. Chem., 1932, 208, 182.
8. Windaus, A., and Bohne, A., Uber Hyo-glyko-desoxycholsaure und uber Hyo-desoxy-cholsaure. Liebig's Ann. der Chem., 1923, 433, 278.
9. Wieland, H., and Reverey, G., Untersuchungen uber die Gallensauren. XXI. Mitteiling. Zur Kenntnis der menschlichen Galle. 1. Ztschr. f. physiol. Chem., 1924, 140, 186.
10. Wieland, H., and Seibert, W., Untersuchungen uber die Gallensauren. LVII. Mitteilung. Uber die Trennung der Inhaltsstoffe der Rindergalle. Ztschr. f. physiol. Chem., 1939, 262, 1.
11. Irvin, J. L., Johnston, C. G., and Anderson, C. E., Effect of oral administration of desiccated hog bile on the bile acid composition of human and canine fistula bile. Am. J. Physiol., 1939, 126, 544.
12. Irvin, J. L., Johnston, C. G., and Sharp, E. A., The enterohepatic circulation of foreign bile acids: the circulation of cholates in hogs with biliary fistulae. Am. J. Physiol., 1946, 146, 293.
13. Irvin, J. L., Johnston, C. G., and Kopala, J., A photometric method for the determination of cholates in bile and blood. J. Biol. Chem., 1944, 153, 439.
14. (*a*) Kaziro, K., and Shimada, T., Uber eine neue Farbreaktion der Desoxycholsäure. Ztschr. f. physiol. Chem., 1938, 254, 57.
   (*b*) Shimada, T., Uber eine Bestimmungsmethode der Desoxycholsäure. J. Biochem. (Japan), 1939, 29, 41.
   (*c*) Shimada, T., Weitere Studien uber die Benzaldehyde-Reaktion der Desoxycholsäure. J. Biochem. (Japan), 1938, 28, 169.
15. Windaus, A., Uber die Konstitution der Hyo-desoxycholsaure. Liebig's Ann. der Chem., 1926, 447, 233.
16. Schmidt, C. L. A., and Dart, A. E., The estimation of bile acids in bile. J. Biol. Chem., 1921, 45, 415.
17. Richey, B. R., A method for regulating pressure in the common duct following choledochostomy. Am. J. Surg., 1940, 48, 609.
18. Walters, W., Greene, C. H., and Fredrickson, C. H., The composition of the bile following the relief of biliary obstruction. Ann. Surg., 1930, 91, 686.
19. Greene, C. H., Walters, W., and Fredrickson, C. H., The composition of the bile following the relief of of biliary obstruction. J. Clin. Invest., 1930–31, 9, 295.
20. Breusch, F., and Johnston, C. G., Zum Verschwinden und Wiedererscheinen der Gallensäuren in der Galle bei vorubergehendem Choledochusverschluss. Klin. Wchnschr., 1934, 13, 1856.
21. Wieland, H., and Jacobi, R., Untersuchungen uber die Gallensauren. XXIII. Mitteilung. Weiteres uber die Zusammensetzung der Galle des Menschen und des Rindes. Zur Konstitution der Anthropodesoxy-cholsaure. Ztschr. f. physiol. Chem., 1925, 148, 232.
22. Colp, R., and Doubilet, H., Differential analysis of bile acids in human gallbladder bile. Arch. Surg., 1936, 33, 913.

23. (*a*) Josephson, B., The circulation of bile acids in connection with their production, conjugation, and excretion. Physiol. Rev., 1941, 21, 463.
(*b*) Josephson, B., and Larsson, H., Elimination of cholic acids. V. Quantity and course of the normal secretion and circulation in man. Acta med. Scandinav., 1941, 107, 584.

24. Whipple, G., and Smith, H. P., Bile salt metabolism. IV. How much bile salt circulates in the body? J. Biol. Chem., 1928, 80, 697.

25. Berman, A. L., Snapp, E., Ivy, A. C., and Atkinson, A. J., On the regulation or homeostasis of the cholic acid output in biliary-duodenal fistula in dogs. Am. J. Physiol., 1941, 131, 776.

THE EFFECT OF GLYCINE ON THE PRODUCTION AND EXCRETION OF URIC ACID[1]

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INTRODUCTION

Although most investigators (1 to 4) have agreed that the ingestion of protein increases the output of uric acid in man, there has been no clear elucidation of the mechanism whereby this is accomplished. Mâres (5) believed that the increased uric acid in urine following high protein feeding resulted from the deterioration of nuclei of digestive cells concerned in the assimilation of the ingested protein. He offered in support of this theory the observation that atropine decreased and pilocarpine increased the output of uric acid. Taylor and Rose (1) found that, although urinary uric acid increased after an intake of high protein, the excretion of urinary creatinine did not change. Lewis, Dunn and Doisy (4) administered various amino acids to men in order to obviate as much as possible the probability of digestive gland activity necessarily involved in converting protein into its constituent amino acids. They found that all amino acids tested except sarcosine increased the output of uric acid. Since this latter amino acid was not catabolized in the body, the authors concluded that the uric acid metabolism of the body was increased by the specific dynamic action of catabolizing amino acids. Rose (6) concluded that the increase in uric acid output after high protein ingestion was due to generalized cellular stimulation occasioned by a higher caloric intake, thus implying that the increase in urinary uric acid was due to some intrinsic protein or amino acid effect. Christman and Mosier (7) gave glycine to subjects and observed an increased urinary uric acid but no change in the renal excretion of creatinine. They apparently concurred with Rose's theory concerning the general stimulating effect of protein or amino acids on cellular metabolism with consequently increased production and secondarily increased excretion of uric acid. Quick (8) also observed that glycine feeding increased uric acid excretion, particularly when combined with salicylate ingestion.

However, various observations have been made which cannot be fitted in with the theory of increased production of uric acid after ingestion of protein or amino acids. Thus, although Lewis, Dunn, and Doisy (4) implied that the specific dynamic action of ingested amino acids effected an increased production of uric acid, they also found that glutamic acid (an amino acid without significant dynamic action) increased uric acid output as markedly as any amino acid tested. Furthermore these investigators in this particular study never determined the concentration of uric acid in blood after ingestion of amino acids—it was assumed apparently that such a rise occurred because of the increased urinary output observed. Later however, Gibson and Doisy (9) were unable to detect a rise in blood uric acid in man after the ingestion of amino acids. Finally, Pitts (10) showed that an excess of an amino acid (glycine) might influence the renal excretion of a blood constituent not by increasing the production of the latter but by competing with it in the renal tubular reabsorptive process. These latter observations suggested the possibility that excess amino acid ingestion might increase urinary uric acid not by initially increasing the concentration of the latter in the blood but by interfering with its renal tubular reabsorption after its glomerular filtration (11).

In order to explore the validity of this last possibility as well as to determine other possible mechanisms involved in the increased excretion of uric acid after ingestion of amino acids, rats were given glycine. Studies then were made of the creatinine, hippurate and uric acid clearances together with the blood uric acid concentrations. Similar studies with the exception of the hippurate clearance were made on 2 human subjects.

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Finally studies were made of the blood concentration of uric acid in nephrectomized rats given glycine. The results of these studies suggested that glycine increased the output of uric acid by effecting initially a decrease in the amount of uric acid reabsorbed by the renal tubules.

METHODS

A. *Animal experiments*

(1) *Clearance and blood uric acid studies*

Nineteen male albino rats, approximately 6 months of age, fed Purina Dog Chow, were used in this study. Control creatinine and hippurate clearances were determined for 2 hours exactly as described in a previous study (12). However, uric acid studies were also made on the rats at the same time by allowing the total period of urine collection to continue past the 2 hours necessary for the above mentioned clearances until a period of 5 hours had been attained. The blood uric acid was determined on the initial and final blood samples taken for the creatinine and hippurate clearances as well as on a third blood sample taken at the end of the 5-hour collection period. The urine collected for 5 hours was analyzed also for uric acid. The blood uric acid clearance was calculated by determining the average uric acid output per hour and dividing this figure by the average blood uric acid concentration per ml. over the 5-hour period (determined by averaging the blood uric acid concentrations of the 3 blood samples taken). In this manner, the creatinine, hippurate, and uric acid clearance as well as the blood uric acid level were determined concomitantly although the uric acid clearance was calculated on a 5-hour collection period. After these control clearances had been made, they were repeated 1 week later on the same rats except that the latter were given 50 mgm. of glycine per 100 grams of body weight at the beginning of the collection period and 5 mgm. every hour for 5 hours. The creatinine and hippurate clearances have been expressed in ml. of plasma per hour per 100 grams of body weight. The uric acid clearance was not found to vary with body weight, hence has been expressed in ml. of blood per hour per rat.

Uric acid in blood was determined according to the method of Folin (13) except that proportionately smaller quantities of reagents were employed in order to determine the uric acid content of no more than 0.5 ml. samples of whole blood. Uric acid in urine was determined according to the method of Benedict and Franke (14).

(2) *Blood uric acid levels in nephrectomized rats*

Seventeen healthy rats were nephrectomized after control blood uric acid determinations had been made. Nine of these rats then received 100 mgm. of glycine by stomach tube immediately after operation and 50 mgm. 3 times a day as long as they lived. The remaining 8 rats received no glycine and served as controls. Blood samples were obtained daily from each rat and analyzed for uric acid. Water but not food was given to the rats.

B. *Human experiments*

Two healthy adult men (W. C., age 34 and M. F., age 36) previously on a purine-free diet for over 48 hours were studied. At 9:30 a.m., each subject was given 2.5 grams of creatinine in 500 ml. of H_2O. At 10:15 a.m., the first blood sample was taken and urine collection begun. Five hundred ml. of H_2O were taken every half hour for 4 hours. At 12:15 p.m. a second blood sample was taken and a final blood sample at 2:15 p.m., at which time urine collection ended. The first two blood samples were analyzed for both creatinine and uric acid but the third sample was analyzed for uric acid alone. One-half ml. of urine was obtained from the collecting jar at 12:15 p.m. and analyzed for creatinine and the urine collected for the entire period of 4 hours was analyzed for uric acid. Thus, a 2-hour creatinine and a 4-hour uric acid clearance were able to be obtained. The 0.5 ml. removed from the collection jar at the end of 2 hours, as in the rat experiments, was included in estimating the total volume (collected over the 4 hours) for the determination of uric acid clearances. Two weeks after these control studies had been made, the entire procedure was repeated on the same 2 subjects except that at the beginning of the collection period, each ingested 25 grams of glycine. Creatinine and uric acid clearances have been expressed in ml. per minute.

RESULTS

A. *Effect of glycine on uric acid output and clearance of the rat*

The administration of glycine to rats over the 5-hour clearance study was found (see Table I) to have increased the uric acid output of 17 of the 19 rats studied. The average uric acid output before glycine ingestion was 0.136 mgm. per hour and 0.170 mgm. per hour afterwards, an increase of approximately 25 per cent. Likewise the average uric acid clearance increased from 10.70 ml. to 14.50 ml. per hour, an increase of 35 per cent.

The average urine volume was observed to decrease (see Table I) from 3.90 ml. to 2.90 ml. per hour after glycine ingestion. The average creatinine clearance however showed no significant change being 34.3 ml. before and 35.4 ml. per hour after glycine had been given. The hippurate clearance also showed no significant change as it was 138 ml. before and 139 ml. per hour after glycine administration. It was clear from these studies then that the increase in uric acid output observed after glycine ingestion was due neither to in-

TABLE I

The effect of glycine on the excretion of uric acid in the rat

Rat	Before glycine					After glycine				
	U.V.[1]	C.C.[2]	H.C.[3]	U.A.E.[4]	U.A.C.[5]	U.V.	C.C.	H.C.	U.A.E.	U.A.C.
98	1.8	14.3	110	.101	6.75	2.9	35.0	—	.173	15.00
75	4.1	46.8	202	.134	10.30	3.5	40.0	146	.168	16.30
29	4.4	44.0	175	.157	14.30	3.7	39.2	157	.206	19.50
72	4.1	34.6	139	.163	15.10	1.3	28.0	180	.175	15.40
50	3.9	35.4	147	.162	11.80	2.4	31.4	—	.187	18.30
43	1.4	14.2	122	.132	9.50	2.7	31.7	—	.210	16.20
52	2.5	24.8	87	.115	12.90	3.5	46.3	—	.176	14.70
42	4.0	35.5	118	.161	15.30	1.2	23.9	—	.199	15.00
01	4.2	18.6	91	.144	9.60	4.1	48.0	—	.151	12.30
89	4.2	34.6	108	.139	9.50	3.2	28.6	119	.127	9.80
21	3.9	34.8	146	.137	10.10	3.4	38.6	—	.175	12.80
88	4.4	43.7	160	.141	9.10	2.7	24.7	108	.208	19.60
53	5.1	34.4	158	.139	9.90	2.5	40.8	142	.178	11.90
29	4.2	28.5	115	.110	9.40	2.5	20.8	64	.184	14.90
55	4.7	48.0	256	.114	8.80	2.9	29.9	160	.138	11.70
90	3.9	38.8	128	.113	9.00	2.6	39.7	129	.128	10.70
02	4.4	32.5	73	.134	9.30	2.9	45.7	81	.125	10.40
39	4.4	50.5	152	.172	10.60	3.6	43.8	191	.158	14.10
77	4.6	37.0	138	.130	12.90	3.6	36.8	192	.164	17.10
Av.	3.9	34.3	138	.136	10.70	2.9	35.4	139	.170	14.5

[1] U. V. equals urine volume in ml. per hour.
[2] C. C. equals creatinine clearance in ml. per hour per 100 grams body weight.
[3] H. C. equals hippurate clearance in ml. per hour per 100 grams body weight.
[4] U. A. E. equals milligrams of uric acid excreted in urine per hour.
[5] U. A. C. equals uric acid clearance in ml. per hour.

creased glomerular filtration nor to increased renal blood flow.

B. *Effect of glycine on blood uric acid of the rat*

Uric acid content of rat's blood was found to increase from 10:00 a.m. to 3:00 p.m. (the hours employed for the clearance studies) in the control rats. Thus (see Table II) the control average blood uric acid was 0.97 mgm. at the beginning of the clearance study, 1.16 mgm. at the end of 2 hours and 1.51 mgm. per 100 ml. at the end of 5 hours. Similarly the blood uric acid was found to increase in those rats given glycine (see Table II), but the increase was not greater than in the control animals. There was little evidence, then, from these results to support the assumption that glycine *per se* increased the *production* of uric acid in the animal body.

The evidence obtained from the nephrectomized rats fed glycine also failed to support the assumption that more uric acid was produced or mobilized in the blood stream following increased intake of amino acid. Thus the average blood uric acid level of 8 control rats before nephrectomy was 1.85 mgm. per 100 ml. of blood. Twenty-four hours after nephrectomy the blood concentration was 1.88 mgm. and 1.96 mgm. per 100 ml. after 48 hours. This represented an increase of 6 per cent in the blood uric acid 48 hours after nephrectomy. The average blood uric acid content of 9 rats given glycine following nephrectomy was 1.71 mgm. per 100 ml. before and 1.80 mgm. and 1.84 mgm. per 100 ml., 24 and 48 hours, respectively, after nephrectomy, representing an increase of but 8 per cent. In short, glycine feeding did not significantly increase the blood uric acid of nephrectomized rats. It must be remembered, however, that the rat converts uric acid into allantoin. It is therefore possible that any excess uric acid produced in these nephrectomized rats may have been converted so quickly to allantoin that no rise in blood uric acid occurred. Accordingly, the experimental data obtained on these nephrectomized rats are not necessarily applicable to human subjects.

C. *The effect of glycine on uric acid output and clearance of man*

The ingestion of 25 grams of glycine by 2 subjects was found to increase the uric acid output of both. The average uric acid output (see Table

TABLE II

The effect of glycine on the blood uric acid of the rat

Rat	Normal rat			Normal rat given glycine*		
	10:00 a.m.	12:00 n.	3:00 p.m.	10:00 a.m.	12:00 n.	3:00 p.m.
	Uric acid	Uric acid	Uric acid	Uric acid	Uric acid	Uric acid
	mgm. per cent	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
98	1.30	1.60	1.60	0.97	1.10	1.40
75	1.20	1.30	1.40	0.94	1.30	0.86
29	0.81	1.10	1.40	1.10	0.97	1.10
72	0.95	1.00	1.30	0.83	1.50	1.10
50	0.92	1.30	1.90	0.94	0.91	1.20
43	1.00	1.30	1.80	1.00	1.10	1.80
52	0.81	0.95	0.92	0.90	1.10	1.60
42	0.75	1.00	1.40	1.00	1.20	1.80
01	0.84	0.95	1.40	1.10	1.30	1.30
89	0.75	0.92	1.50	1.00	1.30	1.60
21	0.84	0.73	1.30	1.00	1.20	1.90
88	1.10	1.00	2.00	0.68	1.00	1.50
53	1.20	1.20	1.60	0.61	1.40	2.50
29	0.95	1.00	1.40	0.90	1.80	1.90
55	1.00	0.96	1.60	0.65	1.10	1.80
90	0.77	1.50	1.50	0.97	1.40	1.20
02	1.40	1.50	1.40	0.90	1.20	1.50
39	0.97	1.80	2.10	0.87	1.20	1.30
77	0.93	1.00	1.10	0.92	0.97	1.00
Av.	0.97	1.16	1.51	0.91	1.21	1.44

* Each rat given 50 mgm. of glycine per 100 grams body weight at start and then 5 mgm. every hour of the 5-hour study period.

III) was 23.40 mgm. before and 39.95 mgm. per minute after the administration of glycine (an increase of 71 per cent). Similarly, the average uric acid clearance increased from 11.90 ml. to 17.50 ml. per minute (an increase of 47 per cent).

As was observed in the rats, the average urine volume also decreased (see Table III) from 3.26 ml. before to 2.49 ml. per minute after glycine. The average creatinine clearance also decreased from 116.0 ml. before to 100.0 ml. per minute after glycine had been given. Similar again to the rat study, no significant change was observed in the blood uric acid level after glycine had been given.

DISCUSSION

The above results suggest quite strongly that glycine does not increase the production of uric acid in the body of either rat or man. On the other hand it was certain from the observations made that glycine ingestion does increase the renal excretion of uric acid, despite the fact that both creatinine and hippurate studies demonstrate the fact that there is neither increase in the rate of glomerular filtration nor in the renal blood flow after glycine. As a matter of fact, the creatinine clearance slightly decreased after glycine in the 2 human subjects tested. These facts lead to the inescapable conclusion that glycine in some manner probably impedes tubular reabsorption of the uric acid contained in tubular fluid. In other words, uric acid output is increased after glycine, not because the blood contains more uric acid nor because the kidney filters or secretes more of it, but because the renal tubule does not reabsorb the usual quantity of uric acid in the presence of excess glycine. The latter may compete with uric acid for reabsorption at the identical tubular site. This mechanism may be quite similar to that postulated by Pitts (10) to explain the preferential tubular reabsorption of glycine with impedance of creatine reabsorption when both are in the glomerular filtrate. Such a mechanism would explain also the fact that, in the present study, there was not the expected change in the rat's creatinine clearance commensurate with the decrease in urine volume (12)—a phenomenon which suggests increased water reabsorption by the tubule.

TABLE III

The effect of glycine on blood uric acid and uric acid excretion in man

Subject	Blood uric acid			U.V.[1]	C.C.[2]	U.A.E.[3]	U.A.C.
	10:00 a.m.	12:00 n.	3:00 p.m.				
	mgm. per cent						
Before glycine							
M. F.	3.20	3.10	3.10	3.95	115	19.20	10.30
W. C.	3.40	3.40	3.50	2.56	118	27.60	13.50
Average:	*3.30*	*3.25*	*3.30*	*3.26*	*116*	*23.40*	*11.90*
After glycine[5] (25 grams)							
M. F.	3.60	3.60	3.90	2.50	100	35.60	16.10
W. C.	4.00	3.90	3.90	2.48	101	44.30	18.90
Average:	3.80	3.75	3.90	2.49	100	39.95	17.50

[1] U. V. equals urine volume in ml. per minute.
[2] C. C. equals creatinine clearance in ml. per minute.
[3] U. A. E. equals milligrams of uric acid excreted in urine per minute.
[4] U. A. C. equals uric acid clearance in ml. per minute.
[5] Glycine given after the 10:00 a.m. blood sample had been obtained.

The failure of the blood uric acid to decrease in the presence of this increased renal output of uric acid may well be due to the ability of the extravascular tissues to maintain the blood uric acid concentration despite its increased renal excretion.

SUMMARY

The administration of glycine to the rat and to man was found to increase the renal excretion of uric acid without a concomitant change in either the renal hemodynamics or in the concentration of uric acid in the blood. Furthermore, glycine feeding was not found to increase the uric acid content of nephrectomized rats' blood.

These findings suggested the probability that glycine increased the excretion of uric acid by impeding its renal tubular reabsorption.

The author wishes to express his thanks for the technical assistance given to him by: Barbara Trousdale, Francis Greenberg, William Cano, Dr. Morton Garfield, and Zora Gross.

BIBLIOGRAPHY

1. Taylor, A. E., and Rose, W. C., The influence of protein intake upon the formation of uric acid. J. Biol. Chem., 1914, 18, 519.
2. Mendel, L. B., and Stehle, R. L., The rôle of the digestive glands in the excretion of endogenous uric acid. J. Biol. Chem., 1915, 22, 215.
3. Benedict, S. R., Uric acid in its relations to metabolism. Harvey Lectures, 1915, Series XI, 346.
4. Lewis, H. B., Dunn, M. S., and Doisy, E. A., Studies in uric acid metabolism. II. Proteins and amino acids as factors in the stimulation of endogenous uric acid metabolism. J. Biol. Chem., 1918, 36, 9.
5. Mares, F., Der physiologische protoplasmastoffwechsel und die purinbildung. Arch. f. d. ges. Physiol., 1910, 134, 59.
6. Rose, W. C., The influence of food ingestion upon endogenous purine metabolism. J. Biol. Chem., 1921, 48, 563.
7. Christman, A. A., and Mosier, E. C., Purine metabolism. II. The effect of the ingestion of glycine on the excretion of endogenous uric acid. J. Biol. Chem., 1929, 83, 11.
8. Quick, A. J., The relationship between chemical structure and physiological response. IV. Conjugation of salicylic acid with glycine and its action on uric acid excretion. J. Biol. Chem., 1933, 101, 475.
9. Gibson, H. V., and Doisy, E. A., A note on the effect of some organic acids upon the uric acid excretion of man. J. Biol. Chem., 1923, 55, 605.
10. Pitts, R. F., A renal reabsorptive mechanism in the dog common to glycine and creatine. Am. J. Physiol., 1943, 140, 156.
11. Bordley, J., and Richards, A. N., Quantitative studies of the composition of glomerular urine. VIII. The concentration of uric acid in glomerular urine of snakes and frogs determined by an ultramicroadaptation of Folin's method. J. Biol. Chem., 1933, 101, 193.
12. Friedman, M., The creatinine, inulin and hippurate clearances in the rat. Am. J. Physiol., 1947, 148, 387.
13. Folin, O., An improved method for the determination of uric acid in blood. J. Biol. Chem., 1930, 86, 179.
14. Benedict, S. R., and Franke, E., A method for the direct determination of uric acid in urine. J. Biol. Chem., 1922, 52, 387.

ELECTROPHORETIC ANALYSIS OF ANTE- AND POSTMORTEM SERUM IN DIFFERENT DISEASES [1]

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Our present knowledge of the protein components of normal human plasma has been markedly advanced in recent years as a result of the blood fractionation program carried out by Cohn and his group (1). To a considerable extent the success of this program was due to the availability of large quantities of pooled plasma from normal humans. In attempting to extend the available information on normal human plasma to pathological human plasma, one is faced with the serious technical difficulty of being restricted to relatively small samples from a patient with a given disease. It therefore seemed desirable to investigate the possibility of using postmortem blood obtained at autopsy. The chief question to be answered in such a study is that of the influence of postmortem conditions on the plasma protein composition. Scudder (2) in a study of various factors influencing blood preservation stated that a serum and plasma sample obtained at autopsy were both "abnormal" on electrophoretic analysis. No reference was made as to the antemortem clinical status, nor were any antemortem analyses carried out. In view of this report it seemed important to determine what changes, if any, were to be found in samples of postmortem blood in different diseased states. Essentially the problem was that of obtaining a sample of blood antemortem and a second sample postmortem and comparing the electrophoretic patterns. A study of 9 cases is reported in the present paper, the results of which indicate that postmortem blood, which may be obtained in considerable quantity, shows no striking electrophoretic variation from that observed antemortem during the course of the disease.

METHODS

Collection of blood samples

At times varying from 8 to 240 hours before death, 25- to 30-ml. samples of blood were collected and allowed to clot at room temperature in a 50-ml. centrifuge tube. After retraction of the clot had begun, the tube and contents were centrifuged at 3,000 r.p.m. for 10 minutes. The clear serum was transferred to a 50-ml. Erlenmeyer flask which was then placed on its side in a quick-freeze unit. Blood was again collected at times varying from ½ to 13 hours after death and treated in the same manner as the antemortem samples. A number of different methods were used to obtain the postmortem blood. If the antecubital veins were in good condition it was found that by placing a tourniquet below the antecubital fossa and allowing the arm to drop off the side of the table with the opposite shoulder elevated so that the back was perpendicular to the table, 25 to 30 ml. could be procured from the distended veins. Frequently the veins in this region were not suitable and thus it was necessary to resort to either cardiac puncture or puncture of the femoral vessels. In cases of necropsy within 3 hours postmortem, the following procedure was found effective for obtaining large quantities of blood (1,500 to 2,500 ml.). The vena cava was incised and a length of tubing 10 mm. in diameter inserted and directed toward the heart. This was secured by tying a ligature about the vessel. The superior portion of the vena cava was either tied or clamped. The distal end of the tube was placed in a suitable container, and the body then elevated so that the feet were at the top of an incline of approximately 40°. It was found convenient to include a glass T-tube near the distal end of the tube to permit application of suction in instances where clots interfered with free drainage.

Electrophoretic analysis

The frozen samples were thawed out at room temperature. The resulting solutions were centrifuged and diluted with 2 parts of 0.10μ sodium diethylbarbiturate buffer, pH 8.6. The diluted samples were then dialyzed against 2 liters of buffer at 1 to 2° C. for 48 hours. The dialyzed samples were examined in the electrophoresis apparatus using the Schlieren scanning technique of Longsworth (3). Electrophoresis was carried out in 11-ml. cells for 150 minutes at a potential gradient of about 6 volts per cm. at 1.5° C. Relative composition of the serum samples was estimated by measuring the areas under the projected patterns after the areas were resolved into a series of symmetrical curves. In all instances, the percentage composition represents the average of the ascending and descending boundaries.

Mobilities were determined by measuring the migration distances of the different components from the starting

[1] Aided in part by a grant from the Wisconsin Alumni Research Foundation.

boundaries. Conductivity measurements were made on the protein-free buffer used as the dialyzing solution.

Total protein was determined by the micro-Kjeldahl technique.

RESULTS

Examination of Table I reveals that in only 1 instance (case 5) was there any significant change in the composition of the postmortem samples as compared with the antemortem samples. It appears from the analyses in case 5 that there is a postmortem decrease in beta globulin and an increase in gamma globulin. Whether this is actually due to a quantitative change in these components or to some unknown qualitative change causing a portion of the beta globulin fraction to migrate as gamma globulin is not apparent. The fact that the postmortem sample in this instance was taken only 1 hour after death suggests that the change is not due to postmortem conditions as such. It should be noted that the average values of the ratios of ante and postmortem analyses are remarkably close to unity.

With the exception of case 9 the total protein determination ante- and postmortem agreed quite closely. The high postmortem value found in this instance is in all probability an artefact due to dehydration of the sample in the freezer.

The serum protein patterns in several of the conditions studied showed marked variations from normal. The range of normal values obtained with sera from young normal adults and determined with the same technique is given in Table II.

TABLE I

*Per cent composition of ante- and postmortem serums**

Case no.	Diagnosis	Hours	Total protein	Alb.	Ratio**	α-1	Ratio	α-2	Ratio	β	Ratio	γ	Ratio
1	Angiomatous mesothelioma of pelvis	59.0	4.53	31.40	1.17	15.90	0.96	19.20	1.05	18.00	1.05	15.50	0.72
		1.5	4.89	26.90		16.50		18.20		17.10		21.30	
2	Agranulocytosis with terminal lobar pneumonia	11.0	6.97	46.10	1.03	12.50	1.02	16.20	0.94	14.50	0.90	10.70	1.07
		3.0	6.99	44.60		12.20		17.20		16.00		10.00	
3	Carcinoma of esophagus with metastases	36.0	7.25	26.40	1.04	8.90	0.87	16.40	0.86	24.60	1.17	23.70	0.96
		13.0	7.86	25.20		10.20		19.00		21.00		24.60	
4	Nonlipoid histiocytosis (Letterer-Siwe's disease)	13.5	7.06	28.00	0.95	12.40	0.85	15.20	1.00	16.10	1.03	28.30	1.11
		2.5	6.84	29.40		14.40		15.20		15.60		25.40	
5	Localized Hodgkins with infiltration of lungs	57.0	7.02	40.30	0.97	9.70	1.03	19.70	0.83	17.90	1.92	12.40	0.76
		1.0	7.52	41.60		9.40		23.50		9.30		16.22	
6	Arteriosclerosis, generalized	7.5	6.58	44.40	1.01	13.10	1.00	12.30	0.82	17.40	1.10	12.80	1.00
		2.0	6.13	43.60		13.00		14.90		15.70		12.80	
7	Myelogenous leukemia	55.0	6.78	51.40	0.94	9.80	1.11	10.00	0.97	16.10	1.22	12.70	0.94
		0.5	6.55	54.30		8.80		10.30		13.10		13.50	
8	Hypernephroma with metastases	135.0	6.91	23.30	1.01	11.30	0.98	13.40	1.01	21.10	0.83	31.90	1.12
		1.00	6.61	22.90		11.50		13.20		24.00		28.40	
9	Hypertensive and arteriosclerotic heart disease, multiple pulmonary emboli	239.5	6.86	42.70	1.23	8.70	1.14	12.40	0.84	19.90	0.84	16.30	0.84
		3.0	8.50	34.70		7.60		14.70		23.70		19.30	
	Mean of ratios:				1.04		1.00		0.92		1.12		0.95

* In each instance top line represents antemortem and bottom line postmortem values.
** Ratio of antemortem and postmortem values.

FIGURE 1

Electrophoretic Diagrams

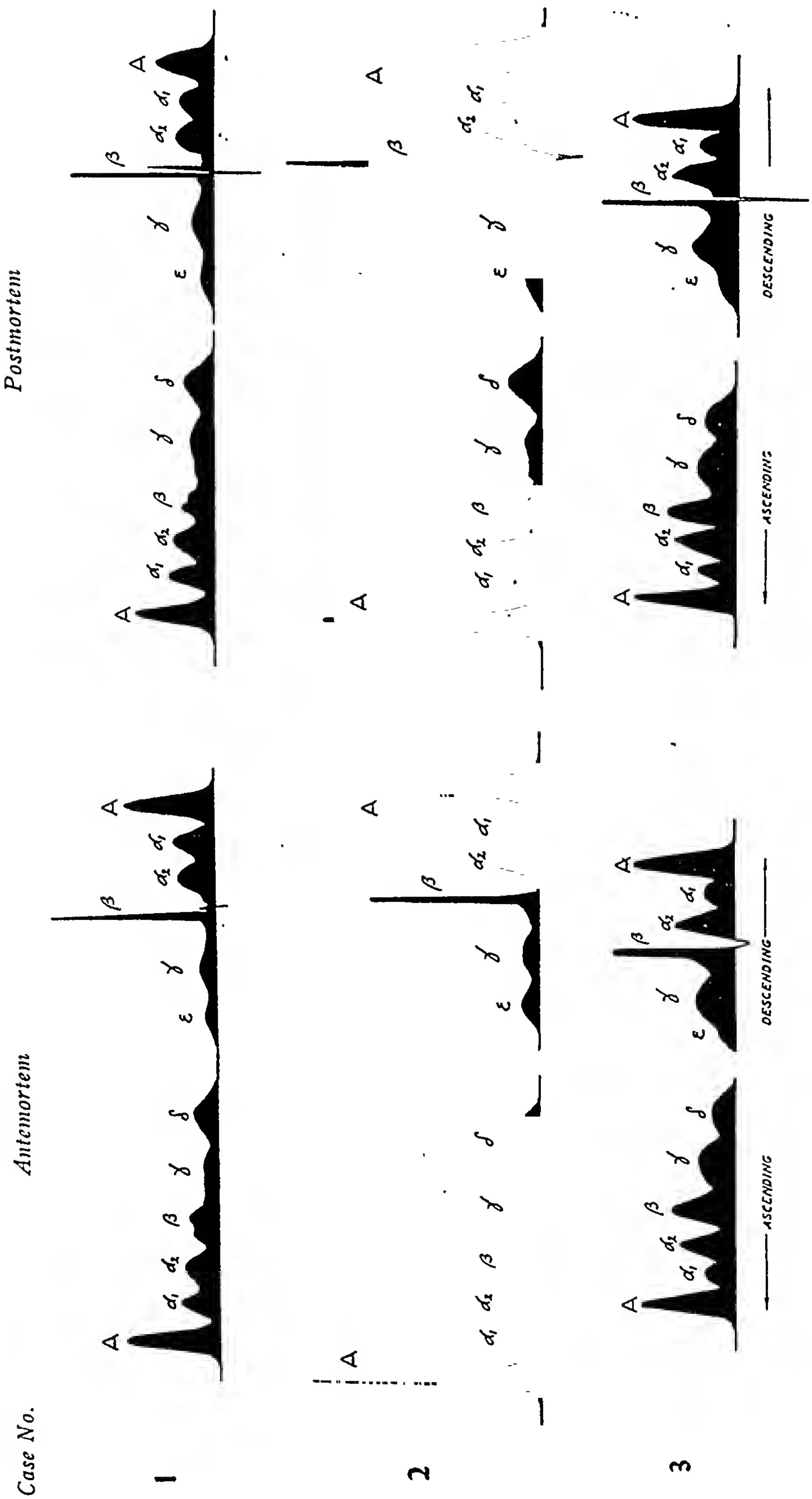

FIGURE 1—*Continued*

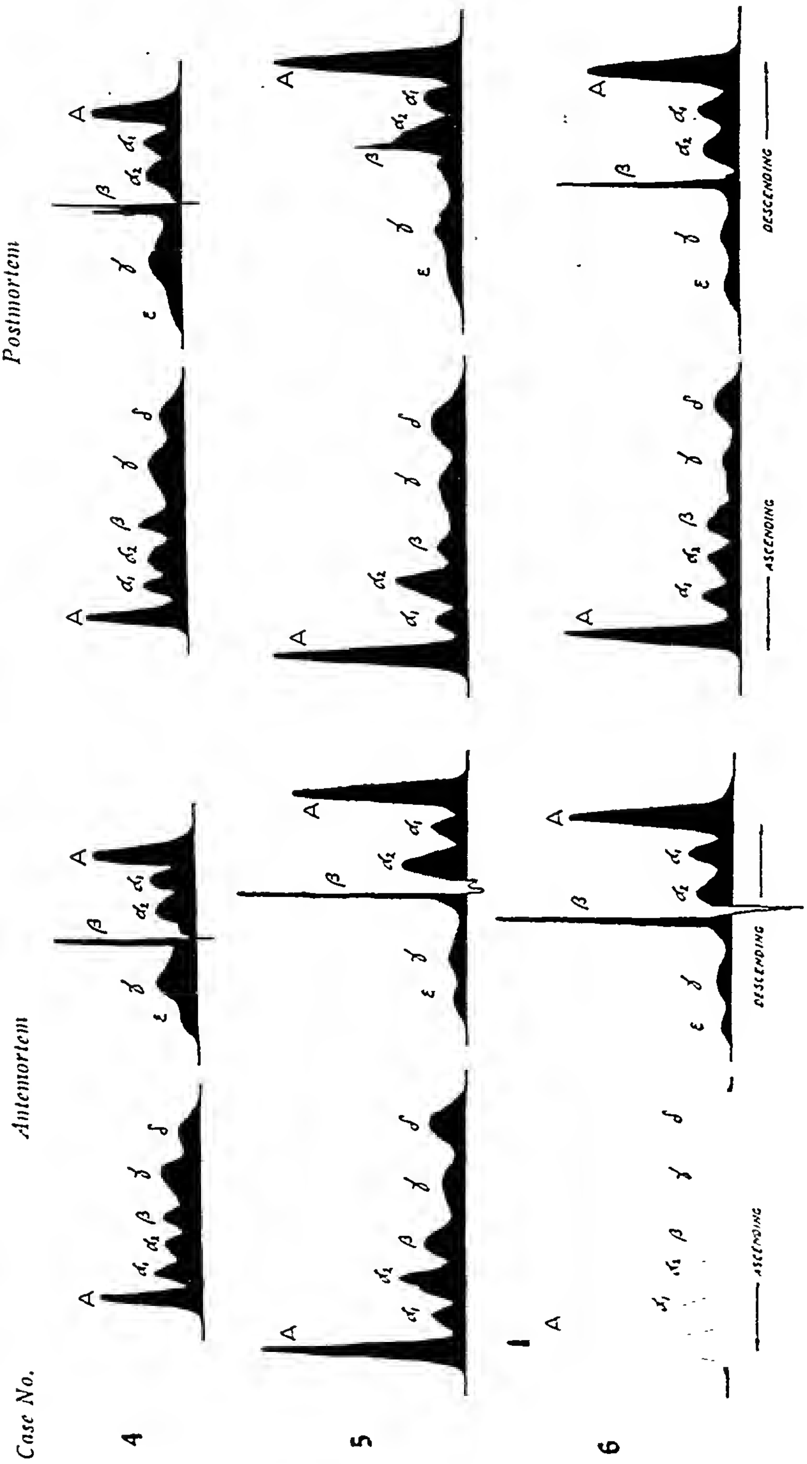

FIGURE 1—*Continued*

TABLE II

Electrophoretic analysis of 10 sera from young normal adults

	Albumin	Alpha-1	Alpha-2	Beta	Gamma
1	53.1	5.4	11.4	16.6	13.8
2	57.8	6.0	8.5	13.7	13.8
3	55.5	5.9	9.8	13.7	15.1
4	54.1	5.0	9.1	16.6	15.2
5	53.1	4.9	8.6	17.2	16.2
6	59.8	4.8	8.0	14.6	12.8
7	58.6	4.8	7.5	13.7	15.4
8	59.0	4.2	9.6	16.6	10.6
9	54.6	5.5	9.3	14.2	16.4
10	59.0	5.2	7.8	15.2	12.8
Mean	56.5	5.2	9.0	15.2	14.2
S.D.	2.8	1.7	1.4	1.5	1.8
S.E. of mean	0.9	0.6	0.5	0.5	0.6

In cases 1, 3, 4, and 8 the serum albumin levels are extremely low. Of interest, however, is the fact that the globulin components are not uniformly increased. Thus, in case 1, marked increases are seen only in the alpha-1 and alpha-2 fractions; in case 3, in all the globulin fractions; in case 4, in the alpha-1, alpha-2, and gamma globulin fractions; and in case 8, in the alpha-1, beta, and gamma fractions. The one fraction showing the most consistent and striking variation from normal in all the conditions studied here is the alpha-1 globulin. This finding is in keeping with the observations made previously by Shedlovsky and Scudder (4).

Tracings of the electrophoretic patterns ante- and postmortem of the different cases and that of a typical normal serum are shown in Figure 1. The greatest apparent variation in the patterns of the ante- and postmortem specimens is seen to occur in the beta-anomaly of the descending patterns. However, variations of the same degree and type have been observed in determinations on a series of normals and even in a series of 3 determinations on the same individual at varying intervals of time.

The mobility values of the different fractions of the normal, antemortem, and postmortem series are shown in Table III. It is apparent that the protein components in serum from cases of disease, ante- and postmortem, have the same mobility values as those found with normal serum.

TABLE III

Mobilities ($cm.^2/volt\ sec. \times 10^{-5}$)

		Albumin	Alpha-1	Alpha-2	Beta	Gamma
Normal	Mean	6.8	5.8	4.6	3.3	1.5
	S.D.	0.23	0.25	0.19	0.22	0.21
Antemortem	Mean	6.8	5.7	4.5	3.2	1.4
	S.D.	0.30	0.30	0.32	0.29	0.26
Postmortem	Mean	6.7	5.7	4.5	3.4	1.5
	S.D.	0.30	0.32	0.31	0.32	0.28

SUMMARY

Serum samples taken ante- and postmortem from 9 cases of different diseases were examined electrophoretically. While all the cases showed some variation from a series of normals, no striking electrophoretic differences were noted in the postmortem as compared with the antemortem samples. On this basis it would appear that up to 13 hours postmortem, serum proteins undergo no significant changes. Thus, the possibility presents itself of preparing purified protein fractions from postmortem blood of diseased patients and comparing their physical, chemical, and biological properties with those obtained from normal pooled plasma.

BIBLIOGRAPHY

1. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, S. H., Jr., The characterization of the protein fractions of human plasma. J. Clin. Invest., 1944, 23, 417.
2. Scudder, John, Studies in blood preservation. The stability of the plasma proteins. Annals of Surg., 1940, 112, 502.
3. Longsworth, L. G., Recent advances in the study of proteins by electrophoresis. Chem. Rev., 1942, 30, 323.
4. Shedlovsky, Theodore, and Scudder, John, A comparison of erythrocyte sedimentation rates and electrophoretic patterns of normal and pathological human blood. J. Exper. Med., 1942, 75, 119.

THE PLASMA NON-PROTEIN NITROGEN DISTRIBUTION AND ITS CORRELATION WITH THE EFFICACY OF FLUID REPLACEMENT THERAPY FOLLOWING THERMAL INJURY[1]

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It has been previously pointed out by us (1) that the rise of the undetermined and the amino nitrogen in the plasma of scalded rats is directly related to the severity of the burn. In the present paper it will be shown that the extent to which the increases in these two nitrogen fractions are reduced by therapeutic measures provides an index of efficacy of the fluid and salt treatment. The therapeutic agencies under comparison were physiological saline, gelatin, albumin, and plasma, which were administered intravenously to the scalded rats at varying intervals of time and in varying quantities. In addition to the effect of these substances upon the azotemia, their influence upon the urinary excretion of water and nitrogen was investigated.

EXPERIMENTAL METHODS

The standardized back-burn procedure, the collection of blood, and the determination of non-protein nitrogen, urea nitrogen, and amino nitrogen in tungstic acid filtrates of the plasma have been previously described (1, 2). The difference (non-protein nitrogen − [urea nitrogen + amino nitrogen]) will be referred to as the undetermined plasma nitrogen.

Plasma protein levels were calculated by multiplying the difference (total plasma nitrogen − non-protein nitrogen) by the factor 6.25. The estimation of the gelatin content of plasma following the infusion of this plasma substitute was based upon the fact that 3 per cent trichloracetic acid precipitates only the plasma proteins while tungstic acid removes both plasma protein and gelatin. The gelatin solution used for infusion contained 1.0 gram of precipitable nitrogen per 5.5 grams of gelatin. Hence the term (non-protein nitrogen of the trichloracetic acid filtrate − non-protein nitrogen of the tungstic acid filtrate) × 5.5 provides an estimate of the gelatin level of plasma.

Urine was collected under "Klarol"[2] in separatory funnels[3] which were placed under metabolism cages. The funnels were drained for measurement and analysis of the urine at intervals of 12, 21, or 24 hours as required. Analyses of total nitrogen, non-protein nitrogen,[4] urea nitrogen, and ammonia nitrogen were carried out with the method of Sobel, Mayer, and Gottlieb (3). The difference (non-protein nitrogen − [urea + ammonia nitrogen]) will be referred to as the undetermined urinary nitrogen.

During the first 24 hours following scalding the rats received no food. Thereafter they were fed in separate cages for periods of 2 hours in the morning and 1 hour in the afternoon, a procedure recommended by Croft and Peters (4). Urine voided during the periods of feeding was absorbed in filter paper. The paper was then extracted with N/50 sulfuric acid and an aliquot of the extract analyzed for urea + ammonia nitrogen. The nitrogen content and the volume of the 3-hour specimens were computed on the assumption that the nitrogen partition and the nitrogen concentration were identical with those of the corresponding 21-hour specimens of known volume and composition.

The urine of rats contains normally a small amount of protein (5). Only during the first day or 2 following the burn, however, were the total nitrogen values of the samples distinctly above the non-protein nitrogen values. Hence, for collection periods other than the first 48 hours following burn, non-protein nitrogen analyses were often omitted and only the total nitrogen determined in addition to urea and ammonia nitrogen.

The following agents were used in the transfusion experiments: (*1*) 0.85 per cent sodium chloride solution referred to thereafter as saline; (*2*) 4 per cent gelatin solution (Knox P-20). In addition to 726 mgm. of nitrogen precipitable with tungstic acid, 100 ml. of the gelatin solution contained 12.5 mgm. of undetermined nitrogen, 2.5 mgm. of amino nitrogen, 1.25 mgm. of ammonia nitrogen and 0.63 mgm. of urea nitrogen, the latter prob-

[1] Part of the results have been presented at a Meeting of the Physiological Society of Philadelphia on October 15, 1946 (Am. J. Med. Sci., 1946, 212, 755).

[2] A very light mineral oil manufactured by Sonneborn and Son, N. J.

[3] In glass funnels filled with a non-polar medium, such as mineral oil, water droplets stick rather tightly to the glass walls and are only incompletely removable mechanically. Perfect drainage was obtained when, on the suggestion of Dr. K. C. Blodgett of the General Electric Research Laboratory, the funnels were coated with a water repelling surface film. We are grateful to the Chemical Department of the General Electric Company, Schenectady, N. Y., for a gift of DRI-FILM No. 9987.

[4] The urines w[illegible] deproteinized with tungstic acid.

ably derived from arginin; (3) 5 per cent human albumin solution containing 1.06 per cent of sodium chloride. This infusion fluid was prepared by adding 4 parts of saline to 1 part of a 25 per cent solution of normal human serum albumin (Lederle) containing 1.8 per cent of sodium chloride; (4) plasma from citrated or heparinized rat blood. The curative effectiveness of both types of rat plasma was identical. Infusion intervals and volumes are given in the figures and tables.

The standard for computing mortality rates was 10 days' survival. The rates referred to in the following sections were obtained in separate experimental series which will be discussed elsewhere (6).

EXPERIMENTAL RESULTS

Effect of fluid replacement on azotemia

Figure 1 illustrates the effect of the intravenous infusion of saline on the non-protein nitrogen levels in the plasma of scalded rats. It is seen that a single infusion, when administered 2 hours after the burn in an amount corresponding to 2 per cent of the body weight, failed to retard the increase of the plasma nitrogen. Repetition of the infusion at 5 hours after the burn prevented a further rise of the undetermined and the amino nitrogen fractions and reduced the mortality rate of the group to 50 per cent. In the survivors examined 24 hours after the burn, these 2 nitrogen fractions had returned to approximately normal level. When the saline infusion was increased to 10 per cent of the body weight of the animal, approximately normal levels were found as early as 12 hours after the burn and all animals survived. It should be noted that the urea nitrogen levels fell much more slowly. Normal urea levels were not obtained until 48 hours after the scalding. This slow reduction in the presence of effective therapy is in keeping with our previous conclusion (1) that there is no definite correlation between the accumulation of urea in the plasma and the severity of the scald or the prognosis.

For comparing the therapeutic action of plasma and colloidal plasma substitutes with that of saline, an arrangement was needed where saline therapy was only of limited value. For this purpose the 30 per cent surface-35-second scald was chosen, which renders the animals less responsive to saline therapy than does the 45 per cent surface-15-second scald. The volumes of the different agents administered corresponded to 4 per cent of the body weights of the animals, the maximum amount that could be given in the case of colloidal solution without leading to undesirable large increases of the circulating blood volume. Under these circumstances the survival rate following saline therapy was 29 per cent.

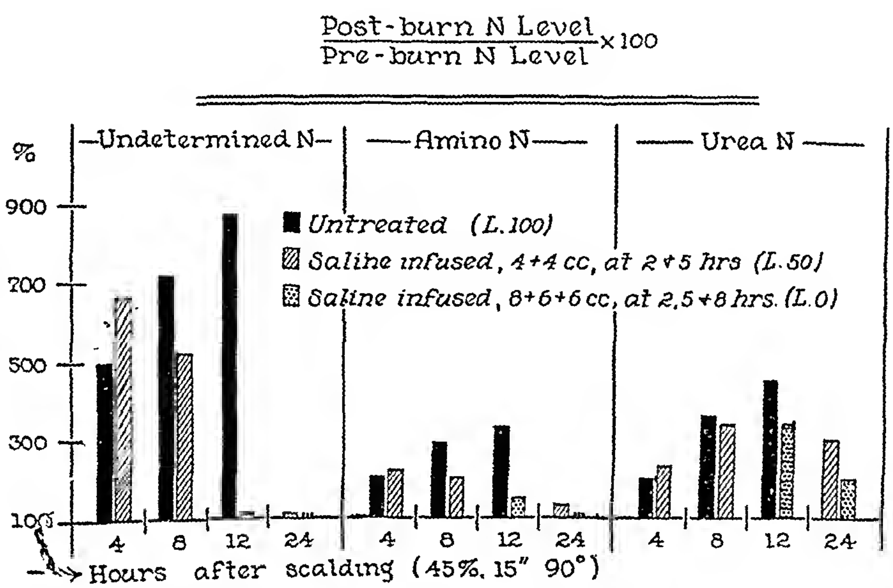

FIG. 1. EFFECT OF THE INFUSION OF VARYING VOLUMES OF SALINE ON THE NON-PROTEIN NITROGEN LEVELS OF SCALDED RATS

Forty-five per cent body surface, 90° C., 15 seconds' scald. Pooled blood from 2 to 4 rats was used for the individual determinations. The nitrogen levels are represented as percentages of the average levels in normal rats. The letter L denotes the average percentile lethality in the animal series.

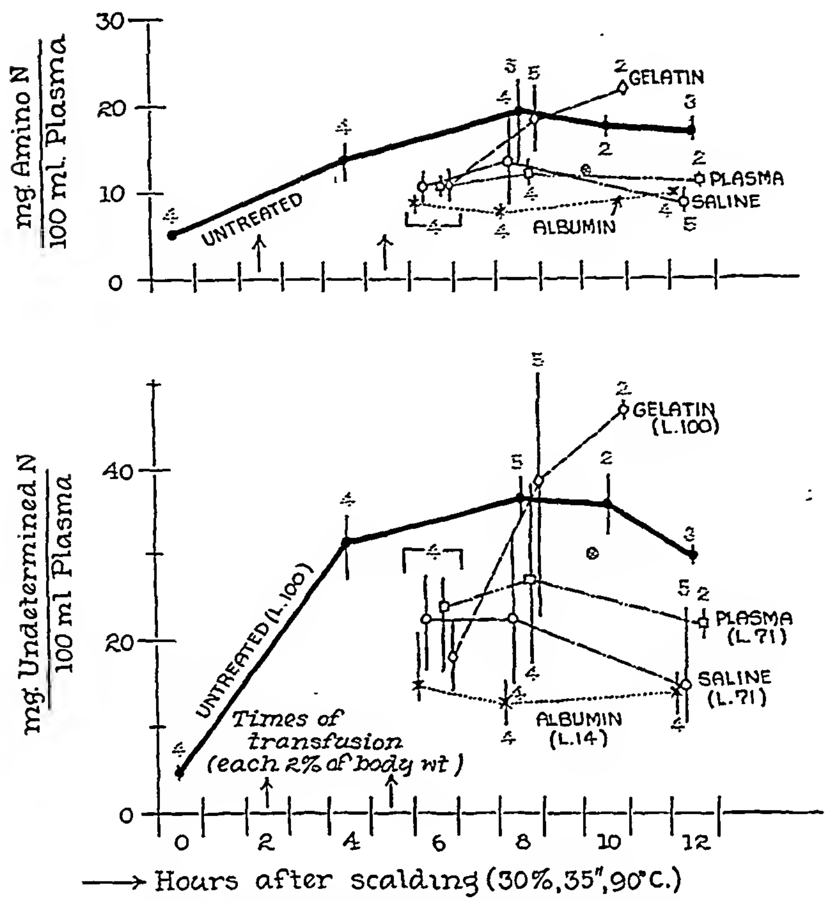

FIG. 2. AMINO NITROGEN AND UNDETERMINED NITROGEN LEVELS IN THE PLASMA OF SCALDED RATS FOLLOWING DIFFERENT TYPES OF FLUID REPLACEMENT THERAPY

Scald: 30 per cent body surface, 90° C., 35 seconds. The analyses were done upon plasma from individual rats.

Note that in the diagram the units of time as marked on the abscissa are represented by the distances between the pairs of vertical lines instead of by points. Within the vertical columns thus formed the values for each of the 5 experimental groups are plotted at arbitrary, but constant, positions with reference to the abscissa. This arrangement prevents the overlapping of the 5 plots. The points of the curves show the group means for each interval of time. The light vertical lines through these points indicate the difference between the highest and the lowest individual analysis while the numerals refer to the number of rats examined. The encircled daggers designate the analyses in an individual moribund rat following albumin treatment. This value has been omitted from the general plot of the albumin group.

A comparison of the plasma nitrogen values with the mortality rates (L) presented in Figures 2 and 3 shows that the response of the undetermined nitrogen levels brought out most strikingly the differences in efficacy of the various types of fluid replacement therapy. It is seen from Figure 2 that albumin infusion, which yielded the lowest mortality rate, was most effective in permanently reducing the undetermined nitrogen levels. This is contrasted to the transient effect of gelatin infusion which did not produce a single cure. Normal rat plasma and physiological saline solution

had an intermediary position with reference to the reduction of the undetermined nitrogen levels and to the lowering of the mortality rates.

The amino nitrogen levels, which are also given in Figure 2, showed trends similar to those of the undetermined nitrogen levels. The differences between the treated and untreated groups were, however, less pronounced. Only following albumin treatment were the group means significantly below those of the controls during the entire experimental period. Plasma infusion resulted in statistically significant reductions at the 8- and 12-hour post-burn interval while the reduction following saline treatment became significant not before 12 hours following the scalding. Gelatin infusion failed to produce significant reductions at any interval of time. The urea nitrogen levels (Figure 3) were not significantly different in the 5 experimental groups under comparison.

Inspection of the data presented in Table I reveals the following details regarding the undetermined nitrogen levels. Six hours after the burn, *i.e.*, 1 hour after the final infusion, the levels in all treated groups were significantly lower than they had been 4 hours after the burn in the untreated controls. Albumin and gelatin appeared to be slightly more effective than physiological saline

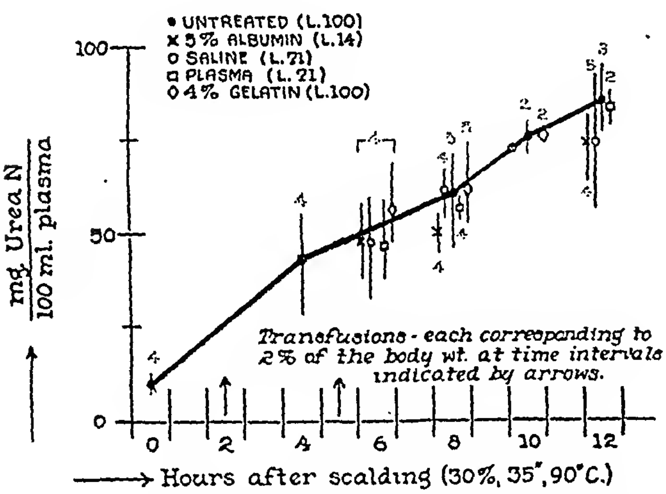

FIG. 3. UREA NITROGEN LEVELS IN THE PLASMA OF SCALDED RATS FOLLOWING DIFFERENT TYPES OF FLUID REPLACEMENT THERAPY

Experimental and diagrammatic arrangements and abbreviations are explained in the legend to Figure 2. Only in the untreated control series have the group means for each interval of time been connected by a curve. None of the group means in the treated series differed significantly from the corresponding control mean.

TABLE I

The effect of fluid therapy on the undetermined nitrogen levels

Group	Time after burn	No. of rats n	Undetermined nitrogen mean values		Change of undetermined nitrogen due to therapy		
			$\bar{x}$	$S\bar{x}$*	$\Delta\bar{x}$	$S\Delta\bar{x}$†	P‡
	hrs.		*mgm. per cent*				
Untreated	4	4	31.9	±1.59			
Gelatin	6	4	18.5	±1.90	−13.5	±2.50	<0.01
Plasma	6	4	23.7	±2.44	− 8.2	±2.95	<0.05
Saline	6	4	22.5	±2.34	− 9.5	±2.86	<0.02
Albumin	6	4	14.9	±2.00	−17.0	±2.75	<0.01
Untreated	8	5	36.4	±0.91			
Gelatin	8	5	38.2	±7.60	+ 1.8	±7.66	>0.05
Plasma	8	4	27.7	±5.10	− 8.7	±5.19	>0.10
Saline	8	4	22.9	±2.64	−13.5	±2.80	<0.01
Albumin	8	4	13.1	±1.28	−23.3	±1.28	<0.01
Untreated	12	3	30.0	±0.55			
Plasma	12	2	22.0	±0.14	− 8.0	±0.57	<0.01
Saline	12	5	15.2	±2.49	−14.8	±2.55	<0.01
Albumin	12	4	14.0	±1.03	−16.0	±1.17	<0.01

* $S\bar{x} = \sqrt{\dfrac{\Sigma(\Delta x)^2}{n(n-1)}}$.

† $S\Delta\bar{x} = \sqrt{(S\bar{x}_u)^2 + (S\bar{x}_t)^2}$ where $S\bar{x}_u$ and $S\bar{x}_t$ designate the standard errors of the means of the untreated and treated groups, respectively.

‡ P = probability of $\Delta\bar{x}$ to have arisen by chance. The P values are taken from Fisher's (7) table of t.

solution and plasma. During the following 2 hours, however, marked differences developed among the 4 groups of infused animals. While the group means for the albumin and saline series remained significantly below that of the untreated controls, the difference between gelatin-treated and untreated animals disappeared and the difference between the plasma group and the controls became statistically insignificant. While the mean for the albumin group was significantly below that of any other treated group, the differences among the 3 other groups were not statistically significant due to the large range of variation of the individual values. The large scatter range in the saline, plasma and gelatin groups can be most readily seen from Figure 2. Since burned rats *in extremis* always exhibited greatly increased undetermined nitrogen levels, heterogeneity of the group can be considered as an indication that in several individuals the beneficial effect of the transfusion had already disappeared and that death was near, although the apparent conditions of most of the treated animals was fair to good at the 8-hour interval of time. During the following 4 hours, however, 70 per cent of the gelatin group, 36 per cent of the plasma group, 20 per

cent of the saline group, and 10 per cent of the albumin group died.

It should be noted that, from the standpoint of alterations from the normal, none of the therapeutic measures can be designated as adequate for the treatment of this particularly severe burn; for, contrary to the 100 per cent successful saline therapy illustrated in Figure 1, all types of fluid therapy failed to reduce to normal the increased undetermined nitrogen levels within a period of 12 hours.

Azotemia and hemoconcentration

Figure 4 serves to demonstrate that gelatin, plasma ,and albumin were about equally effective in relieving the hemoconcentration of burn shock and in maintaining the restored plasma volume. Saline therapy resulted in a temporary hemodilution and hypoproteinemia which disappeared within 3 hours of the last infusion. This was followed spontaneously by a second period of hemodilution with constant plasma protein levels. It is obvious that the effects of these agents upon hemoconcentration do not throw light upon the reasons for their different therapeutic efficacy. Nor can hemodilution *per se* be considered as the essential mechanism in the reduction of the increased undetermined and amino nitrogen levels.

The effect of fluid replacement therapy on water and nitrogen excretion

Table II provides information on urinary nitrogen and water excretion and fluid intake of normal rats during 5 days of feeding and 1 day of

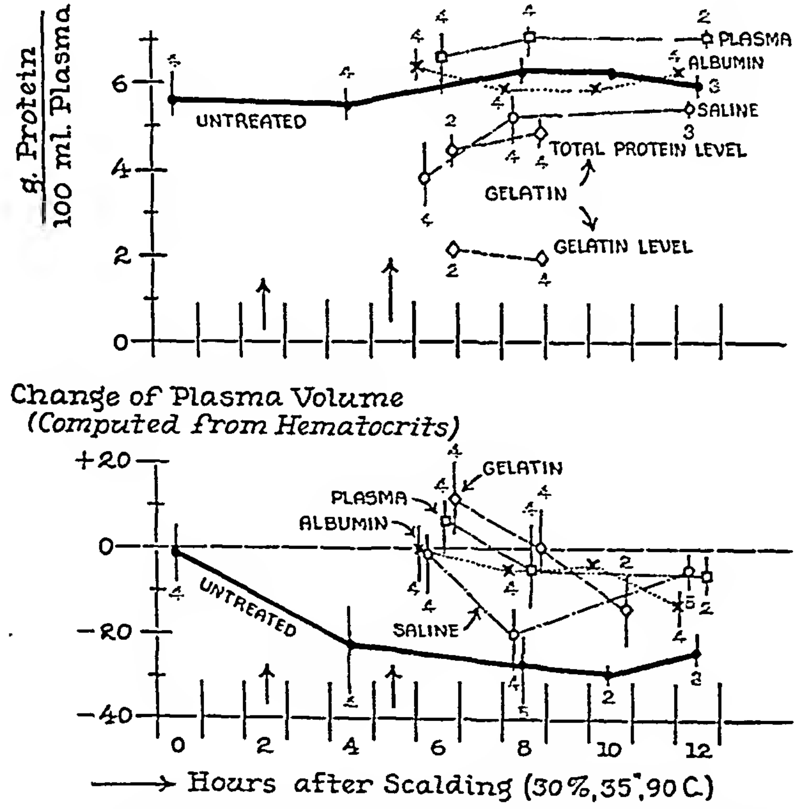

FIG. 4. CHANGES OF PLASMA VOLUME AND PLASMA PROTEIN LEVELS IN SCALDED RATS FOLLOWING DIFFERENT TYPES OF FLUID REPLACEMENT THERAPY

Diagrammatic arrangement as in Figure 2. Hematocrit readings were done upon tail blood. If I is the hematocrit value (expressed as percentage) before the scalding and F the hematocrit reading at the time the animal was sacrificed, then the percentile change (ΔV) of the plasma volume is

$$\Delta V = \frac{[(100 - F) - (100 - I)]\,100}{100 - I} = \left(\frac{I - F}{100 - I}\right) 100.$$

TABLE II

Daily food and water intake, nitrogen excretion, and urine volume prior to the scald

	Nutritional state							
	Fed*				Fast**			
Rat no.⟶	1	2	3	4	1	2	3	4
a. Body weight—*grams*	181.0	196.0	190.5	204.0	172.0	180.0	174.0	186.0
b. Food intake—*grams*	15.6	13.1	14.2	13.8	0	0	0	0
c. Urea + NH_3 N—*mgm.*	214.0	236.0	243.0	249.0	101.0	119.0	115.0	134.0
(c/total N) 100—*per cent*	91.0	89.5	90.0	90.0	83.0	84.0	82.5	84.5
d. Exogenous N = c fed-c fast†—*mgm.*	113.0	117.0	128.0	115.0				
(d/c fed) 100—*per cent*	53.0	49.5	52.5	46.5				
d/b—*mgm. per gram*	7.3	9.0	9.1	8.4				
e. Undetermined N = Total N − c—*mgm.*	20.0	28.0	27.0	27.0	20.0	23.0	24.0	25.0
(e fast/e fed) 100—*per cent*					100.0	82.0	89.0	92.5
f. Water intake—*ml.*	17.4	22.5	23.4	28.8	1.0	1.0	1.0	5.0
g. Urine volume‡—*ml.*	7.2	9.4	7.0	10.4	6.4	10.2	8.6	11.6

* The data are averages for 5 experimental days, 2 before and 3 after the day of fast. The rats were scalded on the fourth day after the fast. Post-burn data are presented in Figure 5.

** Water was withheld for 12 hours in the daytime in order to simulate the post-burn conditions.

† This term should be considered as only a minimal estimate of the exogenous nitrogen because part of the nitrogen excreted during a single day of fast will still be of exogenous origin. Exposure of the rats to longer periods of starvation was not attempted as it would probably have altered their resistance to the scald.

‡ In general, approximately ⅔ of the urine was excreted during the night hours.

fast. Fasting reduced the output of urea + ammonia by approximately 50 per cent while the excretion of undetermined nitrogen and of water was not affected by the nutritional state of the animal. The urea + ammonia excretion during the period of feeding showed the smallest individual variations and the water intake and output the largest.

The experiments upon scalded rats listed in Table III concern the response of urinary excretion to saline therapy corresponding to 10 per cent of the animal's body weight. Data are presented on 2 successive 12-hour periods following the scalding and on 2 types of fatal burn in which the treatment leads to 100 per cent recovery of the animals. It is seen from Table III that in untreated rats, water excretion and, especially urea elimination, virtually ceased. Following the infusion of physiological saline solution the urinary volumes during the first 12 post-burn hours were of a similar order of magnitude as in normal rats during a 12-hour period in the daytime. Abnormal chemical findings in the urines consisted of hemoglobin and low ratios of urea nitrogen to total non-protein nitrogen. The ratio of ammonia nitrogen to non-protein nitrogen, which is not recorded in the table, remained essentially normal (about 6 per cent). During the second 12 hours after the burn, when the animals had access to water, large volumes of urine with a normal nitrogen distribution were excreted. As in fasting normal animals, considerable individual variations were encountered with reference to the quantities of water and nitrogen excreted during the 2 12-hour periods. On the average, the amount of urea + ammonia nitrogen (142 ± 8.2 mgm.) excreted during the first 24 post-burn hours by the group of 6 rats reported in Table II was slightly above the corresponding mean (117 ± 6.5 mgm.) for fasted normal rats. The mean value for the undetermined nitrogen (30.1 ± 3.1 mgm.) was also higher than that (23 ± 1.08 mgm.) for the unburned controls, but this difference was not statistically significant.

Table IV shows the effects of 2 inadequate types of fluid therapy upon the urinary excretion of rats subjected to a 30 per cent surface-35-second scald. As mentioned before, in this type of 100 per cent lethal burn saline infusion corresponding to 4 per cent of the animal's body weight yielded only 29 per cent survival while the infusion of gelatin had no curative value. The saline-treated animals of the experimental series presented in Table IV were in good clinical condition when sacrificed 12 hours after the burn, whereas the gelatin-treated rats were moribund at the time of blood collec-

TABLE III

Urine volume, nitrogen excretion, and plasma nitrogen levels in saline-treated scalded rats

Animals	Interval after burn	Fluid intake	Urine volume	Non-protein nitrogen: In urine volume excreted			Non-protein nitrogen: Per 100 ml. of plasma*		
				Urea $+NH_3$ N	Undetermined N**	Urea $+NH_3$ N / Total NPN	Urea N	NH_2 N	Undetermined N**
	hrs.	*ml.*	*ml.*	*mgm.*	*mgm.*	*per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
Scald: 45 per cent surface, 90° C., 15 seconds									
Mean—4 Rats†	12	0	0.1						
Rat number 1	12	20.4‡	2.4	21.4	17.5	55.0	54.3	11.1	3.8
Rat number 2	12	19.9‡	1.5	16.3	18.1	47.5			
Rat number 2	12–24	27.0	7.0	94.7	8.9	91.5	32.6	7.8	3.3
Mean—6 Rats	12	19.8	2.0	19.3	15.2	55.0			
	12–24	35.0	18.0	121.9	15.4	88.5			
Scald: 35 per cent surface, 90° C., 25 seconds									
Mean—4 Rats†§	12	0	0.2			30.5¶	89.2§	25.4§	25.6§
Rat number 3	12	19.0‡	3.6	71.6	29.1	59.5	54.3	9.1	5.5
Rat number 4	12	22.0‡	4.3	43.4	21.0	49.5	53.8	9.1	6.5
Rat number 5	12	19.0‡	2.5	62.0	16.4	73.5			
Rat number 5	12–24	33.0	31.0	99.1	3.8	96.0	13.2	6.2	1.4

* At the end of the period given in column 3.
** Undetermined nitrogen in urine = non-protein nitrogen—(urea nitrogen + ammonia nitrogen). Undetermined nitrogen in plasma = non-protein nitrogen—(urea nitrogen + amino nitrogen).
† Rats kept in single metabolic cage.
‡ Volume of intravenous saline infusion corresponding to 10 per cent of the animal's body weight. Forty per cent of the total volume were given 2 hours post-burn, the remaining 60 per cent in equal doses 5 and 8 hours post-burn.
§ Three rats died 8 to 9 hours after the scald. The survivor was used for plasma analysis.
¶ Nineteen per cent urea nitrogen and 11 per cent ammonia nitrogen. The non-protein nitrogen concentration was 1.2 grams per 100 ml. of urine.

tion, between 7.5 and 10.5 hours after the burn. Yet the urine volumes of the gelatin-treated rats were within the range previously obtained with corresponding periods of time in scalded rats which received an adequate saline therapy. There was only one statistically significant difference in the urinary excretion following gelatin treatment. The concentration of urea + ammonia nitrogen averaged 0.41 ± 0.05 gram per 100 ml. of urine as contrasted to an average concentration of 0.96 ± 0.10 gram in the urine of 11 saline-treated rats during the first 12 hours following the scalding. This suggests the possibility that the concentration ability of the kidney was impaired in the gelatin-treated rats.

Figure 5 serves to illustrate the nitrogen excretion of saline-treated rats over longer post-burn periods. The same type of scald as in the last experimental series was used, but the infusion was increased to 10 per cent of the animal's body weight with the result that, on the average, 86 per cent of the rats survived. In the graph all quantities are represented as percentages of the corresponding pre-burn values for the fed state which are recorded in Table II. Note that the exogenous urea + ammonia nitrogen excretion is a measure of the food intake of the animal during the post-burn period:

$$\text{d post-burn} = \text{b post-burn} \times (\text{d/b}) \text{ pre-burn}$$

where, as in Table II, d and b designate "exogenous" nitrogen and food intake, respectively. The quantity recorded in Figure 5 is the ratio:

$$(\text{d post-burn/c fed pre-burn}) \times 100$$

c being the total daily excretion of urea + ammonia nitrogen. Prior to the burning, the ratio (d/c fed) × 100 approximated 50 per cent. Hence

TABLE IV

Urinary water and nitrogen excretion in scalded rats infused with saline or gelatin
Scald: 30 per cent body surface, 90° C., 35 seconds

No.	Interval after burn	Urine volume	Non-protein nitrogen: Amount excreted in urine volume: Undetermined N	Urea + NH_3 N	$\frac{\text{Urea}+NH_3\text{ N}}{\text{Total NPN}}$	Plasma concentration: Undetermined N	NH_3 N	Urea N
	hrs.	*ml.*	*mgm.*	*mgm.*	*per cent*	*mgm. per cent*		
Knox P-20 gelatin solution—4 per cent*								
1	7.5	1.6	8.5	5.7	40	50.9	24.9	74.3
2	9.1	1.5	9.5	4.4	31	48.3	22.0	76.1
3	9.2	1.2	8.1	5.8	42			
4	10.6	3.1	22.5	15.2	41	45.8	20.8	75.9
Physiological saline solution*								
5	12.0	2.9	24.8	23.6	49	24.2	9.8	93.0
6	12.0	3.5	26.9	39.2	59	11.9	8.0	80.1

* Infused intravenously at 2 and 5 hours after the scalding. The volume of each infusion (approximately 4.0 ml.) corresponded to 2 per cent of the body weights which ranged from 201 to 215 grams. The total volume (8.0 ml.) infused was approximately equal to the normal plasma volume of the rat.

in Figure 5, the 50 per cent level of the exogenous urea + ammonia nitrogen designates normal food intake.

Rat No. 4 of Table II died 10 hours after the burn in spite of the saline treatment. No urine was excreted, and the plasma nitrogen levels were as high as in the untreated controls. In rats Nos. 2 and 3 the excretion of urea + ammonia nitrogen showed the usual initial drop to the pre-burn fasting level. On the second post-burn day the excretion reached the pre-burn range for fed animals even though food consumption was still minimal. With the resumption of normal food intake (50 per cent level of "exogenous" nitrogen in Figure 5) on the fifth post-burn day, the urea + ammonia nitrogen excretion increased to about 20 per cent above the pre-burn average. It returned to normal the tenth day after the scalding. Rat No. 1 excreted during the first 24 post-burn hours 60 per cent less urea + ammonia nitrogen than during the pre-burn day of fast although the urine volume (13.0 ml.) was as high as in rat No. 3 (11.5 ml.).[5] The nitrogen excretion did not reach the pre-burn level for fed animals before the third day. Food intake remained minimal, and the animal died on the fifth post-burn day.[6]

In the rats Nos. 1 to 3 the daily excretion of undetermined nitrogen averaged 37.0, 33.0, and 42.0 mgm., respectively, during the first 4 post-burn days, *i.e.*, it was 83, 45, and 74 per cent above the fasting level and 83, 19, and 54 per cent above the average level in the fed state. The absolute quantities of undetermined nitrogen excreted were small in comparison with the total amount of urinary non-protein nitrogen. This is in keeping with the statement of Clark, Peters and Rossiter

[5] During the entire post-burn period the average daily urine output of the rats remained 2 to 3 times as high and the average fluid intake double as high as before the scalding. In contrast to the fairly constant rates of nitrogen excretion the daily fluctuations of the fluid exchange were extremely large. This phenomenon has to be re-examined since the possibility could not be excluded with certainty that the rats, whose movements were somewhat impaired owing to scar contractions, spilled drinking water into the collecting funnels.

[6] Because of the sudden death of this animal no blood for plasma analysis was secured. In rat No 2 which was sacrificed at the height of the urinary nitrogen excretion, as well as in rat No. 3 which was analyzed after the return of the nitrogen excretion to the pre-burn range, the non-protein nitrogen levels of the plasma were essentially normal.

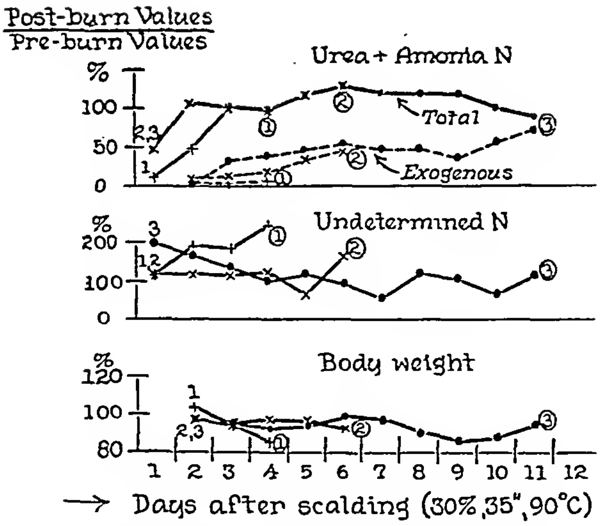

FIG. 5. DAILY NITROGEN EXCRETION AND WEIGHT CHANGES IN RATS RECOVERING FROM BURN AS A RESULT OF SALINE THERAPY

Scald: 30 per cent body surface, 90° C., 35 seconds. The volume of saline infused corresponded to 10 per cent of the animal's body weight. Infusion intervals as in Table III and Figure 1. In the graph all quantities are represented as percentages of the corresponding pre-burn values for the fed state (see Table II). Each curve shows the serial number of the rat to which it refers. Encircled numbers designate the last analysis prior to death or sacrifice of the animal.

(8) that thermal injury in the rat fails to produce the large excretion of undetermined nitrogen which has been reported (9, 10) to occur frequently in the burned human patients. It seems possible, however, that the time-course of the excretion may have some prognostic significance as rat No. 3, which recovered most rapidly from the scalding, showed the greatest initial rate of elimination of this nitrogen fraction.

COMMENT

It has been previously pointed out by us (1) that the accumulation of undetermined and amino nitrogen in the plasma of rats following scalding is probably the consequence of the thermal destruction of cells as well as of the post-burn shock. The latter leads to tissue hypoxia, increased nitrogen catabolism in peripheral tissues, decreased amino acid oxidation in the liver, and cessation of urinary excretion. It should be expected that the curative efficacy of a given type of fluid replacement therapy would be attested by the relief of any one of the 3 criteria of burn shock here examined: the anuria, the hemoconcentration, or the azotemia. While curatively effective types of fluid therapy always initiated the flow of urine and relieved hemoconcentration, curatively ineffective agents, such as gelatin infusion, were not inferior in this regard. They were, however, clearly distinguished from the effective types by the fact that they produced, in all or many animals, only a temporary reduction of the increased plasma levels of the undetermined and amino nitrogen. It seems possible that the renewed rise of the levels of these nitrogen fractions was the manifestation of a returning tissue anoxia, caused by a diminution of the actively circulating rather than of the total blood volume. Recent studies of Prinzmetal, Bergman, and Kruger (11) support the view that the shock following burns is characterized by a pronounced diminution of the circulating blood volume due to an atony of visceral blood capillaries.

SUMMARY

The effect of various types of fluid replacement therapy on the partition of the non-protein nitrogen of plasma and on the urinary water and nitrogen excretion was studied in rats subjected to standardized scalds of known lethality. The agents under comparison were physiological saline solution, gelatin solution, human albumin solution, and rat plasma. The following results have been obtained:

1. The degree of permanent reduction of the increased amino nitrogen and, especially, of the undetermined nitrogen levels of plasma provides a useful criterion of the efficacy of the treatment, whereas the reduction of the urea nitrogen is too slow and too irregular to be of use for this purpose.

2. The rates of urinary water and nitrogen excretion do not furnish a reliable index of curative efficacy. Complete failure of promoting the flow of urine proves, however, inefficiency of the treatment.

3. The effectiveness of fluid replacement therapy in permanently reducing the elevated undetermined nitrogen levels is not clearly related to the relief of hemoconcentration. It is suggested that a renewed rise of the undetermined plasma ni-

trogen levels in spite of nearly normal hematocrit values is an indication of a returning tissue anoxia due to a diminution of the actively circulating rather than of the total blood volume.

BIBLIOGRAPHY

1. Rosenthal, Otto, and McCarthy, M. D., *Post-burn* azotemia, its characteristics and relationship to the severity of thermal injury. Am. J. Physiol., 1947, 148, 365.
2. McCarthy, M. D., A standardized back burn procedure for the white rat suitable for the study of the effects of therapeutic and toxic agents on long-term survival.. J. Lab. & Clin. Med., 1945, 30, 1027.
3. Sobel, A. E., Mayer, A. M., and Gottfried, S. P., A convenient titrimetric ultramicromethod for the estimation of urea and Kjeldahl nitrogen. J. Biol. Chem., 1944, 156, 355.
4. Croft, P. B., and Peters, R. A., Nitrogen loss after thermal burns; effects of adding protein and methionine to the diet of rats. Lancet, 1945, 1, 266.
5. Nelson, V. E., and McCay, C. M., Metabolism and vitamin A. J. Metab. Research, 1925, 7, 199.
6. Rhoads, J. E., McCarthy, M. D., and Parkins, W. M., Personal communication.
7. Fisher, R. A., Statistical Methods for Research Workers. Oliver and Boyd, London, 1938.
8. Clark, E. J., Peters, R. A., and Rossiter, R. J., *Nitrogen metabolism* after burning. Quart. J. Exper. Physiol., 1945, 33, 113.
9. Taylor, F. H. L., Levenson ,S. M., Davidson, C. S., and Adams, M. A., Abnormal nitrogen metabolism in patients with thermal burns. New England J. Med., 1943, 229, 855.
10. Walker, J., Jr., A study of the azotemia observed after severe burns. Surgery, 1946, 19, 825.
11. Prinzmetal, M., Bergman, H. C., and Kruger, H. E., Demonstration of a toxic factor in blood of rats shocked by burn. J. Clin. Invest., 1946, 25, 781.

THE INFLUENCE OF DENITROGENATION ON THE RESPONSE OF ANESTHETIZED DOGS TO INTRAVENOUSLY INJECTED OXYGEN

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INTRODUCTION

A marked clinical improvement in critically ill human subjects after the intravenous administration of oxygen has been reported by a number of investigators (1 to 5) despite the fact that only about 1.5 to 10 per cent of the calculated basal oxygen requirement was injected. However, in normal and in anoxemic experimental animals, other workers (6 to 10) have observed not only rapid, shallow breathing but also decreased rather than increased oxygen content of the arterial blood following the intravenous injection of oxygen at rates of 0.21 to 2.3 ml. per kgm. per minute. The paradoxical anoxemia has been attributed to interference with the pulmonic oxygenation of the blood by the formation of multiple oxygen emboli in the pulmonary capillaries (7). The accompanying respiratory changes, which are abolished by bilateral vagotomy, have been considered (10) as similar to the tachypnea observed in dogs after the production of pulmonary capillary emboli by the intravenous injection of starch granules (11).

Recently, in seeking a simple and effective means of extra-pulmonic oxygenation for supportive treatment in severe, acute pulmonary edema, we became interested in the possibilities of intravenous oxygen administration, and particularly in the cause and prevention of the hypothesized emboli resulting from this procedure. From theoretical considerations, it became apparent that, since the nitrogen tension of the blood and tissues normally is high (573 mm.), gaseous nitrogen present in the blood must enter intravenously injected oxygen bubbles, as in a tonometer. Consequently, if all the oxygen injected were absorbed by the reduced venous hemoglobin before the blood reached the right heart, residual nitrogen bubbles would remain to obstruct the pulmonary capillaries. In recent years, the analogous role of dissolved nitrogen in the bubble formation of decompression sickness and aero-embolism has been demonstrated and the value of prophylactic denitrogenation by inhalation of 99.6 per cent oxygen has been well established (12 to 18). To explore the interrelationship between nitrogen tension and intravascular bubble formation and the anoxemia following intravenously administered oxygen, in the present experiments, comparisons were made between the effects of oxygen administered intravenously to anesthetized dogs without prior denitrogenation and after the nitrogen saturation of the blood and tissues was reduced by continuous intratracheal exposure to 99.6 per cent oxygen before and during the intravenous administration of oxygen.

EXPERIMENTAL

Twenty-six healthy, normal, adult, mongrel dogs weighing from 7.7 to 34.1 kgm. were anesthetized by the intravenous injection of approximately 25 mgm. of sodium pentobarbital per kgm. body weight. A glass, L-shaped cannula was inserted into the exposed trachea and tied in place with heavy, silk ligatures. The femoral artery and vein were exposed bilaterally for blood sampling and for intravenous oxygen administration. The state of anesthesia was maintained by additional pentobarbital injections as required.

Denitrogenation was accomplished by continuous administration for 3 to 4 hours of dry 99.6 per cent commercial oxygen, or occasionally 95 per cent oxygen-5 per cent carbon dioxide, supplied to the animals on demand at low pressures and without re-breathing by means of an Army Air Force, low resistance, A-16 valve, attached to the tracheal cannula by rubber connections as described previously (19). In 3 experiments with very large dogs, the reducing valve leading to the demand valve offered too much resistance at peak inspiratory flows. Therefore, oxygen was supplied from a Douglas-Haldane bag, with the oxygen removed being continuously replaced from a tank connected to the bag.

The intravenously administered oxygen was bubbled through water and, then, continuously injected into the femoral veins through two ½-inch, 27-gauge needles. The rate of the injection was measured by a calibrated, capillary orifice flowmeter interposed between the water bottle and the rubber tube leading to the needles. Respiratory rates were observed before, during, and after

intravenous oxygen administration. Additional details of experimental procedure are given below.

By direct femoral arterial or venous puncture, blood samples were collected in heparinized, air-free syringes, which were immediately sealed with mercury, and stored at 0° C. Determinations of nitrogen content and oxygen content and capacity were performed in duplicate generally by the Edwards-Roughton-Scholander techniques (20, 21). Occasionally, additional checks were made by the usual Van Slyke manometric method (22). In some experiments, nitrogen contents were determined by a shorter manometric method recently described by Horvath and Roughton (23).

In an effort to avoid the pulmonary passive congestion and edema which Drinker (24) has observed in anesthetized dogs after prolonged immobilization, the animals, which were restrained in a supine position when blood samples were drawn or intravenous oxygen was administered, were moved frequently during the experiment. Despite this precaution, however, on autopsy many exhibited some congestion.

Until the day of the experiment, diets consisting of fox chow, milk, horse meat, and water *ad libitum* were permitted.

RESULTS

1. *The effect of intravenous oxygen administration on the arterial oxygen content of denitrogenated dogs*

An initial series of experiments confirmed the observations of others (6 to 10) that oxygen administered intravenously to anesthetized dogs at rates as low as 1 ml. per kgm. per minute (12 to 30 per cent of the basal oxygen requirement) rapidly produced marked increases in respiratory rates and, often, greater than 25 per cent decreases

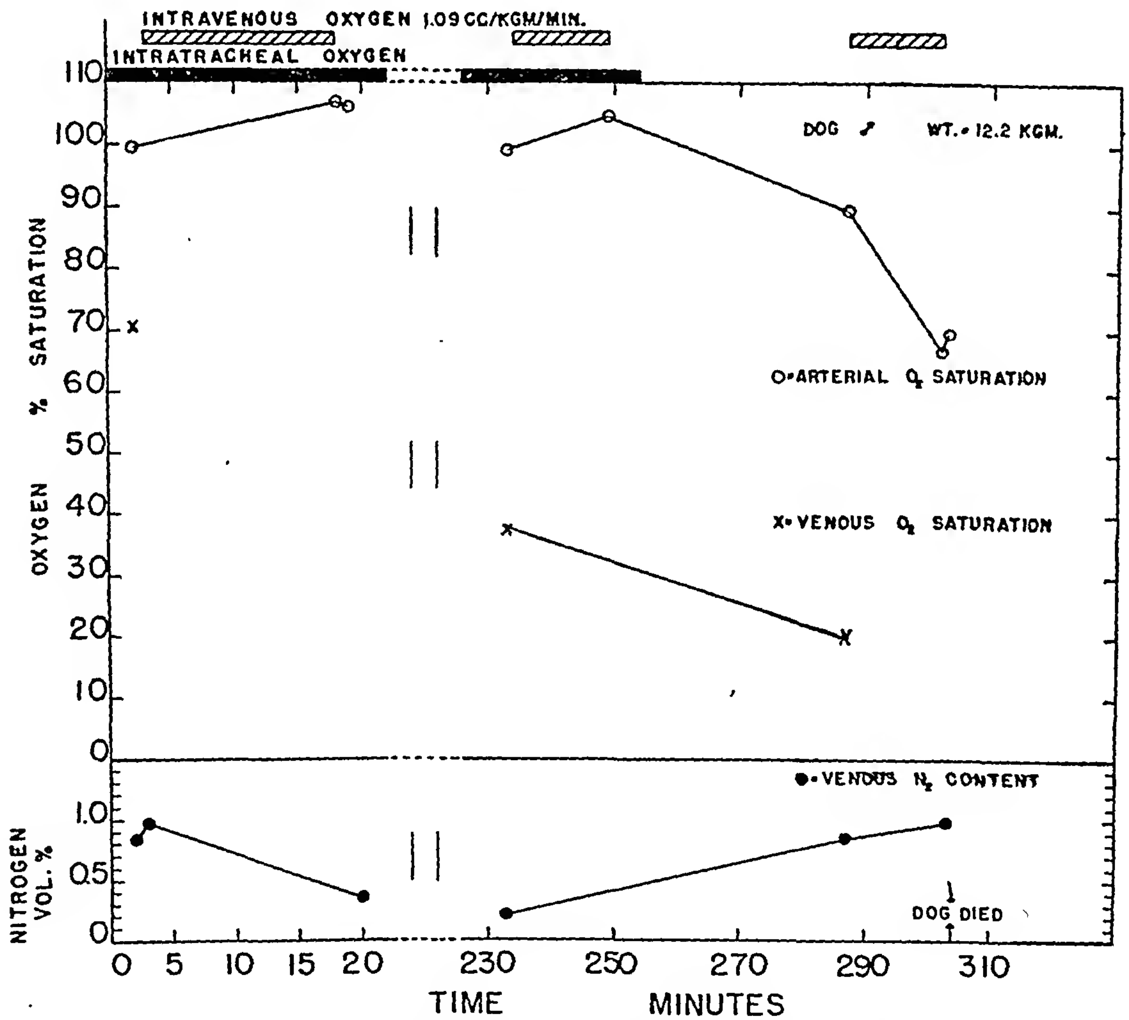

FIG. 1. ARTERIAL OXYGEN SATURATION AND VENOUS NITROGEN CONTENT IN A 12.2 KGM. ANESTHETIZED DOG BEFORE AND AFTER INTRAVENOUS INJECTION OF OXYGEN DURING AND FOLLOWING DENITROGENATION BY CONTINUOUS INTRATRACHEAL ADMINISTRATION OF 99.6 PER CENT OXYGEN

Intravenous oxygen was injected at a rate corresponding to 21.9 per cent of the calculated basal requirement.

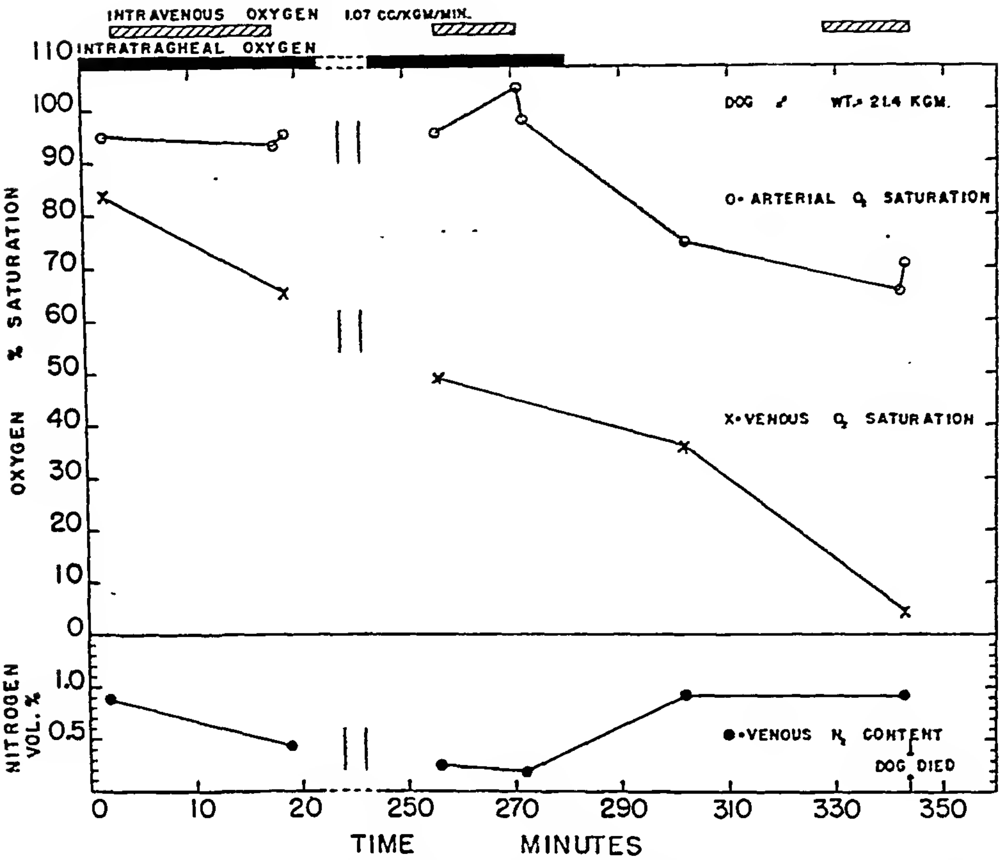

Fig. 2. Arterial Oxygen Saturation and Venous Nitrogen Content in a 21.4 Kgm. Anesthetized Dog Before and After Intravenous Injection of Oxygen During and Following Denitrogenation by Continuous Intratracheal Administration of 99.6 Per Cent Oxygen

Intravenous oxygen was injected at a rate corresponding to 24.3 per cent of the calculated basal requirement.

in arterial oxygen content. A second series of experiments revealed that intravenously injecting 1 ml. of oxygen per kgm. per minute for 20-minute periods in tracheotomized, anesthetized dogs which had been denitrogenated for from 3 to 4 hours, as described above, by exposure to 99.6 per cent oxygen usually produced falls in arterial oxygen content, frequently of the magnitude of 5 volumes per cent, although occasionally there occurred slight increases. Table I gives the pertinent data for 4 representative experiments in which venous nitrogen content was reduced to 0.18 to 0.27 volumes per cent before oxygen (12.5 to 33.1 per cent of the basal requirement) was administered intravenously.

TABLE I

Pertinent data indicating changes in arterial oxygen content following the intravenous injection of oxygen for 20 minutes in tracheotomized, anesthetized dogs, denitrogenated by continuous exposure to 99.6 per cent oxygen for 3 to 4 hours

Dog no.	O_2 Injected Expressed as per cent of basal requirement	Venous N_2 content at time of injection of O_2	Arterial O_2 content: Before injection of O_2	Arterial O_2 content: After injection of O_2
	per cent	*volume per cent*		
1	12.5	0.19	19.6	15.0
2	16.9		19.9	20.8
	17.3		17.5	18.4
3	22.0	0.18	19.6	18.0
4	33.1	0.27	21.0	16.0

2. *Effect of intravenous oxygen on the arterial oxygen saturation in dogs before and after denitrogenation by exposure to 99.6 per cent oxygen*

Binger, Brow, and Branch (11) found that inhalation of 90 per cent oxygen abolished the anoxemia observed in dogs after the production of

multiple pulmonary capillary emboli by intravenous injection of starch suspensions. Therefore, the less marked fall in arterial oxygen content of the denitrogenated dogs following the injection of intravenous oxygen may have resulted not from the prophylactic denitrogenation but from therapeutic effects of the 99.6 per cent oxygen by means of which denitrogenation was achieved and maintained. Consequently, the following experiments were performed to determine whether the simultaneous administration of 99.6 per cent oxygen intratracheally without "complete" denitrogenation would also prevent the development of the anoxemia.

Figures 1 to 4 graphically illustrate 4 representative experiments in which intravenous oxygen was administered at rates of 1.03 to 1.09 ml. per kgm. per minute to anesthetized dogs for three 15-minute periods as follows: (*1*) two minutes after the start of the intratracheal exposure to 99.6 per cent oxygen and, therefore, presumably before denitrogenation was complete; (*2*) after 4 hours of intratracheal oxygenation to achieve marked denitrogenation; and (*3*) if the animal survived the 2 earlier injections, approximately 30 to 60 minutes after discontinuing the intratracheal oxygen to permit the animals to become renitrogenated by breathing air.

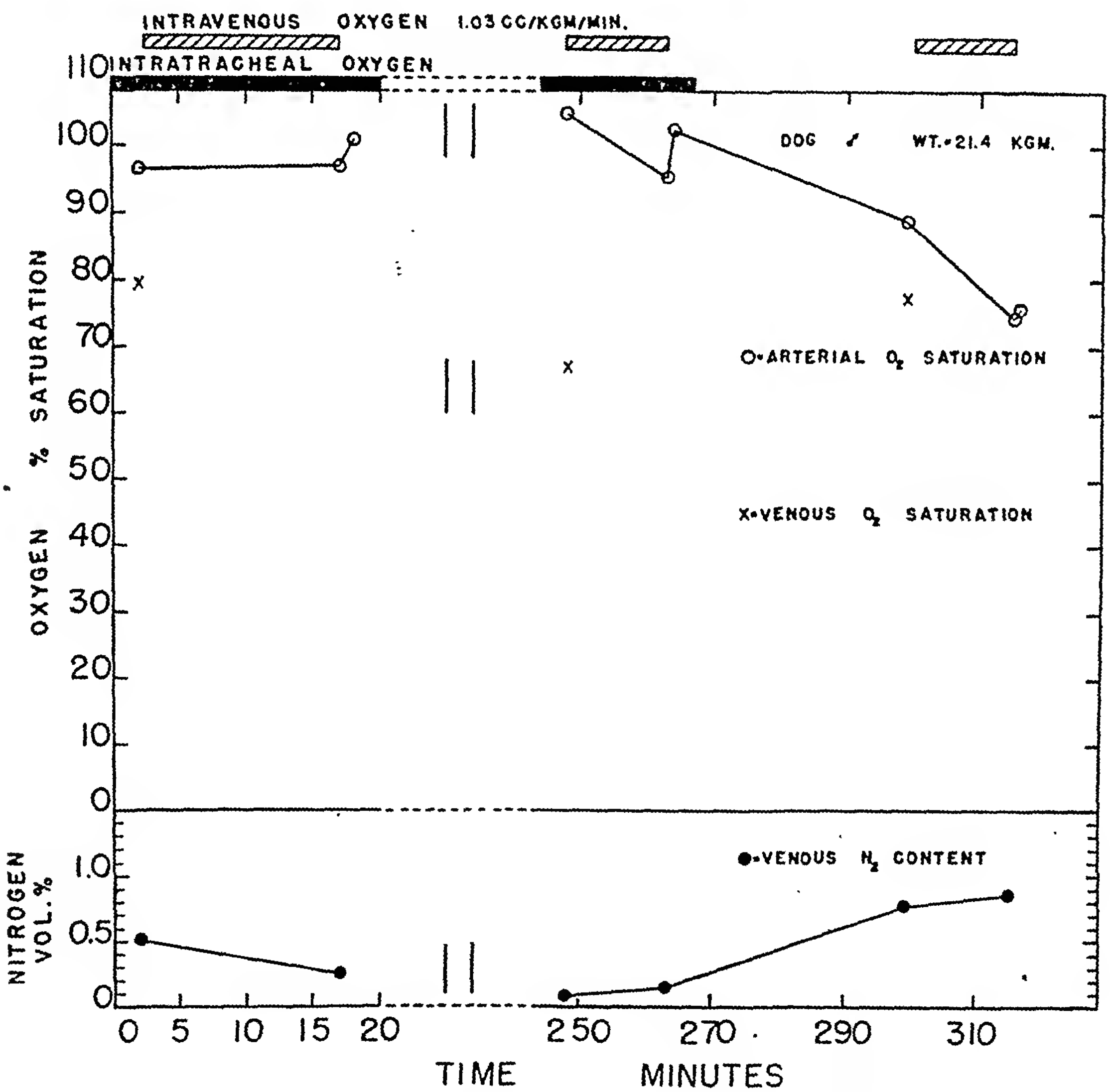

Fig. 3. Arterial Oxygen Saturation and Venous Nitrogen Content in a 21.4 Kgm. Anesthetized Dog Before and After Intravenous Injection of Oxygen During and Following Denitrogenation by Continuous Intratracheal Administration of 99.6 Per Cent Oxygen

Intravenous oxygen was injected at a rate coresponding to 23.4 per cent of the calculated basal requirement.

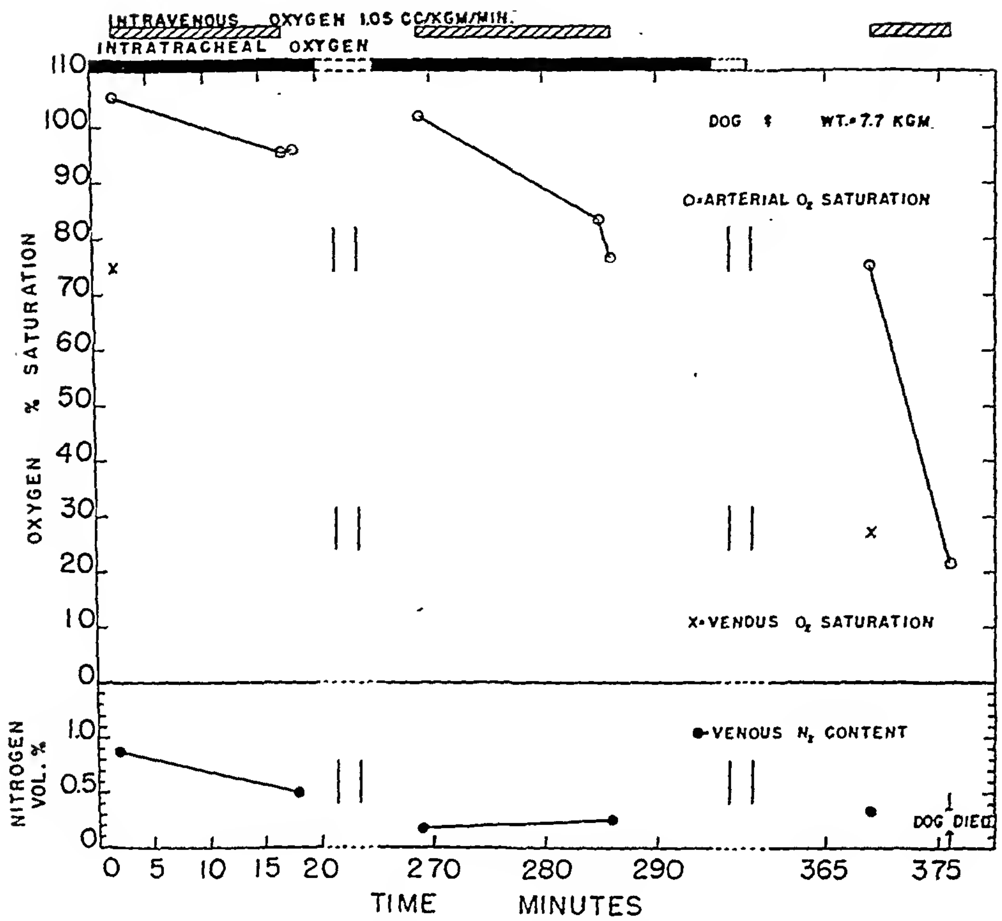

FIG. 4. ARTERIAL OXYGEN SATURATION AND VENOUS NITROGEN CONTENT IN A 7.7 KGM. ANESTHETIZED DOG BEFORE AND AFTER INTRAVENOUS INJECTION OF OXYGEN DURING AND FOLLOWING DENITROGENATION BY CONTINUOUS INTRATRACHEAL ADMINISTRATION OF 99.6 PER CENT OXYGEN

Intravenous oxygen was injected at a rate corresponding to 15.2 per cent of the calculated basal requirement.

In 2 of the animals (Figures 1, 3) administration of intravenous oxygen 2 minutes after the start of intratracheal oxygen resulted in increases in oxygen saturation of the arterial blood. However, in both of these animals, the venous nitrogen content by the end of the 15-minute injection period had fallen to values of 0.36 volume per cent and 0.26 volume per cent respectively. During the first period of intravenous oxygenation in the other 2 animals, there was a fall in the arterial oxygen saturation, and the decrease in venous nitrogen content was not so marked; *i.e.*, it fell from 0.89 and 0.87 volume per cent to 0.43 and 0.50 volume per cent, respectively (*cf.* Figures 2 and 4). Similarly, when the intravenous oxygen injections were repeated after more complete denitrogenation, in 2 of the 4 animals, there was a rise (Figures 1, 2), whereas in the other 2 there was a fall (Figures 3, 4) in arterial oxygen saturation although the nitrogen contents of the venous blood at the start of the injection periods were 0.21, 0.23, 0.09, and 0.15 volume per cent, respectively. Thus, in these experiments, there was no direct relationship between the nitrogen content of the venous blood and the response to intravenous oxygenation. However, the final intravenous injections of oxygen at the same rate, when the dogs were more completely nitrogenated and were breathing not oxygen but air, produced rapid, marked drops in arterial oxygen saturation in 3 of the animals and

a moderate drop in the fourth, with death in 3 occurring within a few minutes after the cessation of the injection (Figures 1 to 4). In all instances, the injection of oxygen resulted in a sharp rise in respiratory rate, the increases being, respectively, from 24 to 120, 40 to 70, 19 to 116, and 26 to 60. It will be noted that the least renitrogenation and greatest fall in arterial oxygen saturation occurred in the dog represented by Figure 4. This animal, at autopsy, showed appreciable congestion of the lungs.

3. *Effect of intravenously injected air on oxygen saturation in dogs*

There was no significant difference in the response to intravenous oxygen of dogs when either more completely denitrogenated or only partially denitrogenated by exposure to intratracheal oxygen for 2 minutes before and during the 15-minute period of injection. As a result, it was not clear whether the lesser drop or slight rise in arterial oxygen saturation following intravenous oxygen injection in both the partially and more completely

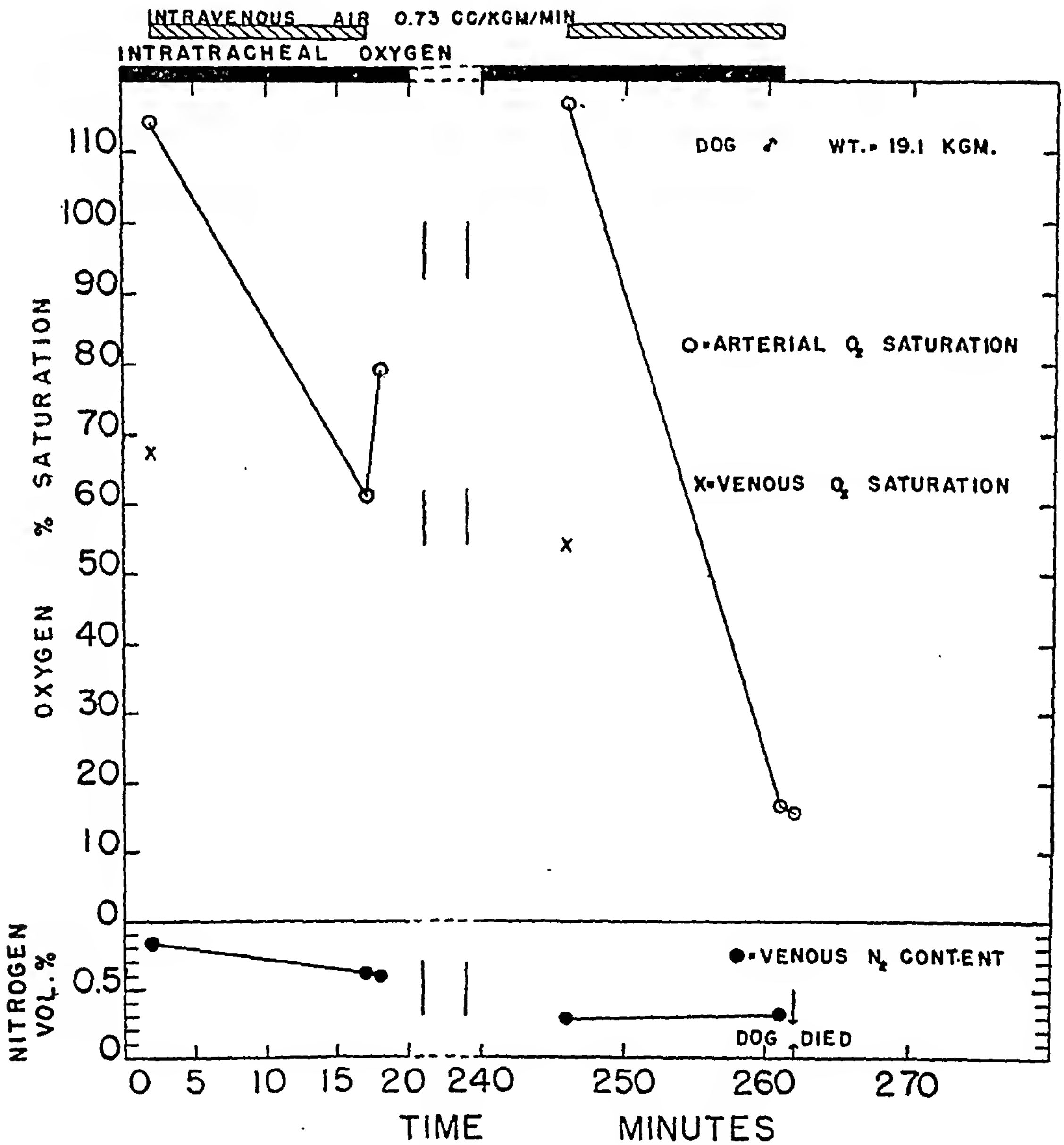

Fig. 5. Arterial Oxygen Saturation and Venous Nitrogen Content in a 19.1 Kgm. Anesthetized Dog Before and After the Intravenous Injection of Air During and Following Denitrogenation by Continuous Intratracheal Administration of 99.6 Per Cent Oxygen

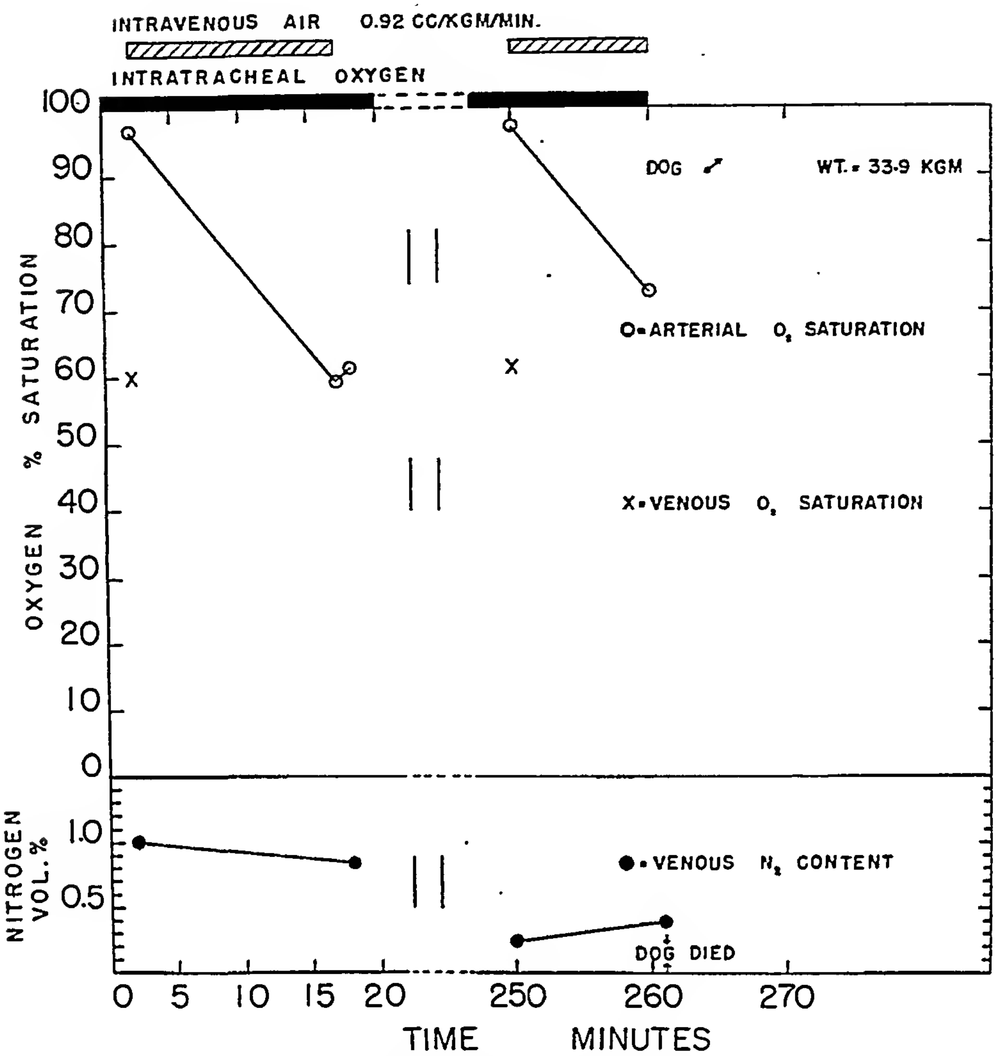

FIG. 6. ARTERIAL OXYGEN SATURATION AND VENOUS NITROGEN CONTENT IN A 33.9 KGM. ANESTHETIZED DOG BEFORE AND AFTER THE INTRAVENOUS INJECTION OF AIR DURING AND FOLLOWING DENITROGENATION BY CONTINUOUS INTRATRACHEAL ADMINISTRATION OF 99.6 PER CENT OXYGEN

denitrogenated dogs was a consequence of the beneficial effects of the prophylactic denitrogenation or of hyperventilation in the presence of the high intratracheal oxygen concentration. If the less severe reactions to intravenous oxygen resulted merely from hyperventilation due to bubble formation in the pulmonary capillaries of animals which were breathing high concentrations of oxygen, similar results should be obtained if the hyperventilation were produced by injection of an inert gas—even nitrogen itself—at comparable rates. Therefore, the previous experiment was repeated in a series of dogs in which air rather than oxygen was injected intravenously.

Figures 5 to 7 illustrate typical experiments in which air was injected intravenously at rates of 0.73, 0.92, and 1.16 ml. per kgm. per minute, respectively. In contrast to the injection of oxygen, the intravenous injection of air during simultaneous administration of intratracheal oxygen produced a rapid and extensive fall in arterial oxygen saturation both before and after considerable lowering of venous nitrogen content had been achieved, despite the marked hyperventilation which oc-

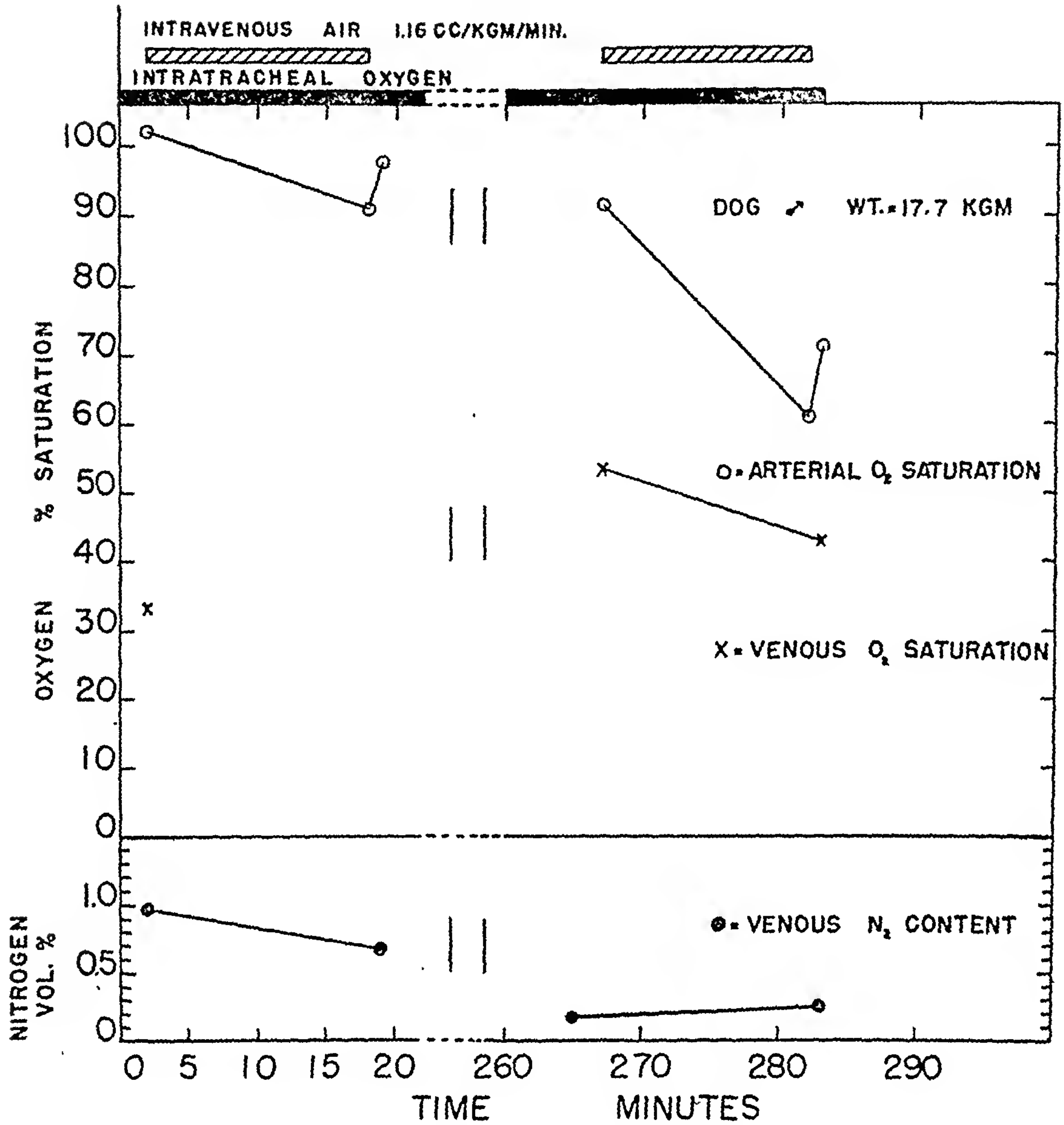

FIG. 7. ARTERIAL OXYGEN SATURATION AND VENOUS NITROGEN CONTENT IN A 17.7 KGM. ANESTHETIZED DOG BEFORE AND AFTER THE INTRAVENOUS INJECTION OF AIR DURING AND FOLLOWING DENITROGENATION BY CONTINUOUS INTRATRACHEAL ADMINISTRATION OF 99.6 PER CENT OXYGEN

curred. In no experiment could the third injection without intratracheal oxygen be made, since the animals all died after the second injection, although in some (*cf.* Figures 5, 6) the intravenous air was injected at a much slower rate than was the intravenous oxygen.

4. *The effect of injecting intravenous oxygen at very slow rates in normal anesthetized dogs without previous denitrogenation*

Inasmuch as there have been reports of marked clinical improvement in anoxemic human subjects who received prolonged intravenous injections of very small amounts of oxygen (1 to 5), arterial and venous oxygen saturations were followed in pentobarbitalized dogs during the intravenous administration of oxygen at comparable rates for periods up to 3 hours. Because of the briefer duration of these experiments, reinforcing doses of pentobarbital were not required to maintain the anesthesia. In Figure 8, 3 such experiments are graphically presented.

In all 3 animals, although the venous oxygen saturation was only 44 per cent to 73 per cent at the start of the experiment, as would be expected in deeply barbitalized animals breathing air, the

intravenous injection of oxygen at rates of 0.082 (A) to 0.345 ml. per kgm. per minute (B) resulted in continued fall of both arterial and venous oxygen saturation. In one experiment (Figure 8, B), the animal died after 150 minutes although the oxygen administered intravenously was only 7.4 per cent of the basal requirement.

The decline in oxygen saturation in all 3 animals and the death of the 1 animal must have been related to the intravenously administered oxygen for the following reasons: (*1*) generally, in pentobarbitalized animals, the blood oxygen saturation does not decrease but increases after several hours as the anesthesia tends to lighten, (*2*) the only other experimental procedure to which these large dogs were subjected was the withdrawal of a total of 30 to 50 ml. of blood over a period of about 180 minutes, and (*3*) at autopsy, no other significant contributory pulmonary pathology was found.

The results of these studies are graphically illustrated in Table II.

TABLE II

Tabular summary of the representative general effects of intravenously administered oxygen and air on the respiration and arterial oxygen saturation of denitrogenated and non-denitrogenated, anoxemic dogs

Substance administered	Denitrogenated dogs			Non-denitrogenated anoxemic dogs		
	Per cent of basal requirement	Respiratory rate	Arterial O_2 saturation	Per cent of basal requirement	Respiratory rate	Arterial O_2 saturation
i.v. O_2	15.2 to 33.1	+	−	1.9 to 7.4	++	−−
i.v. Air	−	+++	−−−	−	+++	−−−

+ = increase.
− = decrease.

DISCUSSION

The better response of dogs to the intravenous injection of oxygen at rates of approximately 1 ml. per kgm. per minute when intratracheal oxygen is administered simultaneously probably is not due solely to the beneficial effects of the inhaled oxygen. It should be recalled that when air was injected intravenously at equivalent or slower rates in dogs which were breathing 99.6 per cent oxygen after more or less denitrogenation, the fall in arterial oxygen saturation was as marked and rapid as that observed in animals given intravenous oxygen while breathing air (Figures 5 to 7, 8). This indicates that the therapeutic effect of

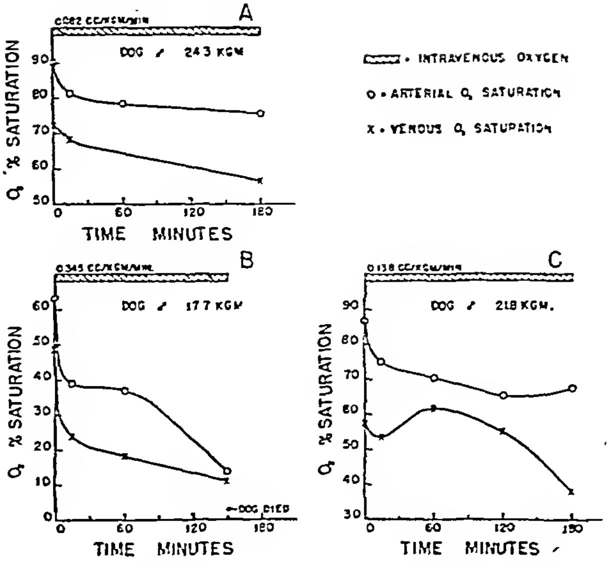

Fig. 8. Arterial and Venous Oxygen Saturation of Anesthetized, Tracheotomized Dogs Breathing Air During the Continuous Intravenous Injection of Oxygen at Rates Which Corresponded to the Following Percentages of the Calculated Basal Requirement: 1.9 Per Cent in A, 7.4 Per Cent in B, and 3.2 Per Cent in C

99.6 per cent intratracheal oxygen alone is insufficient to effect the anoxemia produced by the hypothesized pulmonary emboli. Consequently, some other factor must be involved.

As noted above, Binger, Brow, and Branch (11) found that the administration of 90 per cent oxygen could abolish the anoxemia following production of multiple pulmonary capillary emboli by the intravenous injection of starch granules in dogs. Consequently, because the anoxemia following air injections was not compensated by the simultaneous administration of 99.6 per cent oxygen it could be argued that the air injections do not constitute an adequate control and that the 2 types of experiments reported in the present study differ only in that perhaps more absorption occurred from the oxygen bubbles than from the air bubbles, leaving smaller bubbles in the former case, producing a less severe anoxemia which could be better compensated by the inhaled 99.6 per cent oxygen.

However, because of the demonstrated role of dissolved gaseous nitrogen in the genesis and perpetuation of bubbles in the blood and tissues in decompression sickness and aero-embolism, it is probable that in fully nitrogenated animals, dis-

solved nitrogen similarly enters the injected oxygen bubbles in accordance with Henry's law and that the less severe reaction to this procedure when intratracheal oxygen is simultaneously administered is a consequence of the denitrogenation achieved by the oxygen inhalations. In the denitrogenated dogs, on the other hand, as a result of the lower nitrogen tension, particularly of the blood, probably less nitrogen enters the injected oxygen bubbles which, therefore, may become smaller in size if oxygen is taken up by the reduced hemoglobin in the venous blood. In this regard, it is interesting to note that the initial and final increase in respiratory rate during intravenous oxygen injections in dogs simultaneously inhaling 99.6 per cent oxygen was markedly less than that observed during either intravenous oxygen injections in dogs breathing air or during air injections in dogs breathing 99.6 per cent oxygen, indicating that the oxygen bubbles in the denitrogenated animals possibly were of smaller size or less persistent than in the other animals.

The prolonged injections of intravenous oxygen at very slow rates in anesthetized dogs without denitrogenation reported in the present communication establish further the physiological hazard of this procedure. Despite the slow rate of injection and the initial post-barbiturate anoxemia, arterial and venous oxygen saturation continued to fall or to remain low throughout the injection period, with death occurring in 1 animal before the 180-minute injection period was completed.

Moreover, several incidental experimental observations demonstrate that even with denitrogenation and simultaneous intratracheal oxygen, intravenous oxygen constitutes an added hazard rather than a therapeutic aid in the anoxemic state. In experiments with 3 very large dogs and a smaller reducing valve leading from the oxygen tank to the demand valve, the resistance to respiration was markedly increased at peak inspiratory flows. Consequently, these animals while being denitrogenated by oxygen inhalation became progressively more anoxemic because of the inspiratory resistance which produced an inadequate respiratory flow and, ultimately, pulmonary congestion and pulmonary edema. In one animal, for example, arterial oxygen saturation was 63.5 per cent, venous oxygen saturation was 30.9 per cent, and venous nitrogen content was 0.23 per cent, when the injection of intravenous oxygen at a rate of 1 ml. per kgm. per minute (23.2 per cent of basal oxygen requirement) was begun. Within 14 minutes, respiration had stopped, and at 15 minutes no cardiac sounds were audible. Similar observations made on the 2 other animals indicate, in addition, that the failure of intravenous oxygen administration in the remaining denitrogenated dogs was not necessarily a consequence of any elevation in oxygen saturation of the venous blood, which might result from simultaneous inhalation of 99.6 per cent oxygen.

These experiments establish the practical therapeutic inadequacy of intravenous oxygen administration, even when the effect of gaseous nitrogen in the blood and tissues is minimized by prophylactic denitrogenation. Other considerations also make it evident that until some means of preventing bubble formation is developed, introduction of significant amounts of oxygen by injection into the venous blood will not be successful. For example, if oxygen is injected into peripheral vessels, the amount which can be absorbed rapidly before coalescence of bubbles occurs is limited immediately to that which will saturate fully the venous return from the areas drained. Because the peripheral blood flow is small in comparison with the total cardiac output, Goodwin *et al.* (25) attempted to inject oxygen into the inferior vena cava through a special catheter which produces small bubbles, but had no particular success in preventing the development of the usual signs and symptoms of pulmonary capillary embolization.

Moreover, as Adriani has noted recently (26), intravenously injected oxygen, like air, produces bubbles which are surrounded by a thin protein film through which neither nitrogen nor oxygen can penetrate too readily. Adriani explains the persistence of the emboli by quoting Langmuir (27) who suggested that the films are persistent and resistant, because the protein is denatured. Thus, any oxygen bubble introduced in the circulation will be only slowly absorbed even in markedly unsaturated blood. Therefore, successful extrapulmonic oxygenation probably will require not intravenous injection but some other technique for introducing oxygen into the blood without bubble formation.

Cessation of the intravenous injection of oxygen was followed within 1 minute in a number of instances by a sudden and variable, but non-persistent, rise in the arterial oxygen saturation. A similar phenomenon was observed and is more fully commented upon by Goodwin *et al.* (25).

Because of the observations made by a number of workers regarding oxygen depression, following the inhalation of pure oxygen during anoxemia resulting from deep barbiturate anesthesia, and central nervous system depression (28), the objection may be raised that the intravenous administration of oxygen to anoxemic dogs under pentobarbital anesthesia can depress or completely check respiration. It should be pointed out that although oxygen depression is readily obtained in the intact pentobarbitalized dog, it is accompanied by an almost immediate precipitous fall in respiratory rate and depth, which may happen even after only 1 breath of pure oxygen. In the series of experiments currently being reported, however, the intravenous injection of oxygen to the anoxemic dogs produced not a decrease, but an increase in respiratory rate, suggesting that the stimulus of anoxemia to the carotid and aortic bodies had not been removed.

SUMMARY

1. The observations of others that oxygen administered intravenously to dogs at rates as low as 1 ml. per kgm. per minute rapidly produced marked increases in respiratory rates and often greater than 25 per cent decreases in arterial oxygen content were confirmed.

2. Prior to the administration of intravenous oxygen, animals were denitrogenated in order to reduce the incidence of intravascular bubble formation.

3. In tracheotomized, anesthetized dogs denitrogenated by exposure to 99.6 per cent oxygen for 3 to 4 hours, the intravenous injection of oxygen at a rate of 1 ml. per kgm. per minute for 20-minute periods usually produced falls in arterial oxygen content, frequently of the magnitude of 5 volumes per cent, although occasionally there occurred slight increases.

4. In dogs which were first denitrogenated and then renitrogenated by breathing air, the intravenous administration of oxygen at rates the same as those previously used produced a rapid, significant drop in arterial oxygen saturation in most instances, and, in some cases, death.

5. In contrast to the results obtained on injection of oxygen during the simultaneous administration of intratracheal 99.6 per cent oxygen, the intravenous injection of air under similar conditions produced a rapid, extensive fall in arterial oxygen saturation both before and after considerable lowering of venous nitrogen content had been achieved, despite the marked hyperventilation which occurred.

6. The intravenous injection of oxygen for 180 minutes at very slow rates (0.082 to 0.345 ml. per kgm. per minute) in deeply anesthetized and anoxemic dogs breathing air also resulted in a continued fall of the arterial and venous oxygen saturation, although the amount of oxygen administered represented merely 1.9 to 7.4 per cent of the basal requirement.

7. It is suggested that because of the physiological and physical factors involved in bubble formation, successful extra-pulmonic oxygenation will require some technique by which oxygen can be introduced into the blood without the formation of bubbles.

8. Even with denitrogenation and simultaneous intratracheal oxygen, intravenous oxygen constitutes an added hazard rather than a therapeutic aid in the anoxemic state.

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BIBLIOGRAPHY

1. Neudörfer, A., Zur intravenösen Sauerstoffinfusion. Wien Klin. Wchnschr., 1905, 18, 89.
2. Tunnicliffe, F. W., and Stebbing, G. F., The intravenous injection of oxygen gas as a therapeutic measure. Lancet, 1916, 2, 321.
3. Singh, I., and Shah, M. J., Intravenous injection of oxygen under normal atmospheric pressure. Lancet, 1940, 1, 922.
4. Ziegler, E. E., The intravenous administration of oxygen. J. Lab. & Clin. Med., 1941, 27, 222.
5. Jacobi, M., Klein, B., Rascoff, H., Kogut, B., Auerbach, R. W., and Jennings, J. E., The effect of intravenous administration of oxygen on shock in in dogs and in human beings. Arch. of Surg., 1946, 52, 42.
6. Stuertz, E., Ueber intravenöse Sauerstoffinfusion. Ztschr. f. diatet. phys. ther., 1904, 7, 67.

7. Bourne, G., and Smith, R. G., The value of intravenous and intraperitoneal administration of oxygen. Am. J. Physiol., 1927, 82, 328.
8. Singh, I., Intravenous injection of oxygen with the animal under ordinary and increased atmospheric pressure. J. Physiol, 1935, 84, 315.
9. Dick, M., Respiratory and circulatory responses to intravenous oxygen and their relation to anoxemia. Am. J. Physiol., 1939, 127, 228.
10. Grodins, F. S., Ivy, A. C., and Adler, H. F., Intravenous administration of oxygen. J. Lab. & Clin. Med., 1943, 28, 1009.
11. Binger, C. A. L., Brow, G. R., and Branch, A., Experimental studies on rapid breathing. I. Tachypnea, independent of anoxemia, resulting from multiple emboli in the pulmonary arterioles and capillaries. J. Clin. Invest., 1924, 1, 127.
12. Behnke, A. R., Thomson, R. M., and Shaw, L. A., The rate of elimination of dissolved nitrogen in man in relation to the fat and water content of the body. Am. J. Physiol., 1935, 114, 137.
13. Behnke, A. R., Application of measurements of nitrogen elimination to the problem of decompressing divers. U. S. Nav. Med. Bull., 1937, 35, 219.
14. Behnke, A. R., The absorption and elimination of gases of the body in relation to its fat and water content. Medicine, 1945, 24, 359.
15. Whiteley, A. H., McElroy, W. D., Warren, G. H., and Harvey, E. N., Bubble formation in animals. V. Denitrogenation. J. Cell. and Comp. Physiol., 1944, 24, 257.
16. Whitaker, D. M., Blinks, L. R., Berg, W. R., Twitty, V. C., and Harris, M., Muscular activity and bubble formation in animals decompressed to simulated altitudes. J. Gen. Physiol., 1945, 28, 213.
17. Eggleton, P., Elsden, S. R., Fegler, J., and Hebb, C. E., A study of the effects of rapid decompression in certain animals. J. Physiol., 1945, 104, 129.
18. Whiteley, A. H., and McElroy, W. D., Denitrogenation of muscle and fat tissues of the anesthetized cat. Am. J. Physiol., 1946, 146, 229.
19. Karel, L., and Weston, R. E., The nitrogen content of femoral arterial and venous blood in anesthetized dogs during denitrogenation by continuous inhalation of 99.6 per cent oxygen—In press.
20. Edwards, G. A., Scholander, P. F., and Roughton, F. J. W., Micro gasometric estimation of the blood gases. III. Nitrogen. J. Biol. Chem., 1943, 148, 565.
21. Roughton, F. J. W., and Scholander, P. F., Micro gasometric estimation of the blood gases I. Oxygen. J. Biol. Chem., 1943, 148, 541.
22. Van Slyke, D. D., and Neill, J. M., The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. J. Biol. Chem., 1924, 61, 523.
23. Horvath, S. M., and Roughton, F. J. W., Improvements in the gasometric estimation of carbon monoxide in blood. J. Biol. Chem., 1942, 144, 747.
24. Drinker, C. K., Pulmonary Edema and Inflammation. Harvard Univ. Press, Cambridge, 1945.
25. Goodwin, W. E., Harmel, M., and Lamont, A., Experiments with intravascular gases: oxygen and helium—to be published.
26. Adriani, J., The Chemistry of Anesthesia. Charles C. Thomas, Springfield, Ill., 1946.
27. Langmuir, I., Cold Spring Harbor Symp. Quant. Biol. 1938, 6, 136.
28. Marshall, E. K., Jr., and Rosenfeld, M., Depression of respiration by oxygen. J. Pharmacol. Exper. Therap., 1936, 57, 437.

THE CONJUGATED, NON-PROTEIN, AMINO ACIDS OF PLASMA. IV. A DIFFERENCE IN THE UTILIZATION OF THE PEPTIDES OF HYDROLYSATES OF FIBRIN AND CASEIN

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Peptides are utilized much less readily than free amino acids upon the intravenous infusion of a partial enzymatic hydrolysate of casein (Amigen), as indicated by the greater accumulation and longer persistence of peptides in plasma, and their loss to the extent of 36 to 53 per cent into the urine (1). However, a partial acid hydrolysate of fibrin twice as rich in peptides as Amigen (containing about ⅔ of its amino acids in a conjugated state) has been found to have a high biological value upon intravenous administration to dogs, as compared with fibrin given orally (2). These observations suggest a possibility of considerable interest to the subject of peptide metabolism, namely, that the peptides derived from fibrin may be more efficiently utilized than those of the casein hydrolysate. This we have found to be the case.

Five per cent solutions of the fibrin hydrolysate in 5 per cent glucose were infused intravenously into convalescent male patients and into a normal subject, in 3 different quantities—1,000 ml., 878 ml., and 485 ml.,—the latter 2 quantities supplying according to our analysis the same quantity of total α-amino nitrogen and bound α-amino nitrogen respectively, as a liter of 5 per cent Amigen. (The fibrin hydrolysate was not only much richer in peptides because of the limited hydrolysis, but also somewhat richer in total amino acids.).

With the 2 larger portions, containing 2 to 2.3 times the quantity of peptides supplied by a liter of Amigen, dialyzable amino acid conjugates (peptides [1]) reached concentrations in plasma somewhat higher than for Amigen at similar infusion rates (Table I). However, when the quantities of peptides infused were alike, the plasma levels were lower with the fibrin hydrolysate. The highest level of peptide α-amino nitrogen yet observed, 6.3 mgm. per cent, was not associated with discomfort. The infusion of either hydrolysate brought the free α-amino acid nitrogen of plasma to levels lower than were observed before the infusions. The curves picturing the removal of the conjugates from plasma show the same aspects with the 2 hydrolysates (Figure 1). Without regard to the rate or quantity of the infusion, the loss of peptides into the urine was less for the fibrin hydrolysate, 26 to 28 per cent of the peptides infused (Table II). For infusion times of 1.5 to 4 hours, losses of 40 to 53 per cent were characteristic for the peptides of 1 liter of 5 per cent Amigen. In the 2 cases where the conditions were most similar (experiment 52, reported previously [1], and experiment 14), the same quantity of peptides being infused into the same normal subject in very nearly the same time interval, the loss of peptides was 27 per cent for the fibrin hydrolysate and 53 per cent for the Amigen. The losses of *free* α-amino acids were larger during infusion of the fibrin hydrolysate than when Amigen was injected. Undoubtedly a part of the free amino acids in the urine originated from infused peptides of the hydrolysates. The over-all urinary losses of α-amino acids (free + peptide) of both preparations were in the region of 20 per cent.

The additional conjugates of the experimental urines appeared to be peptides. The optical density (at 515 mμ) given with the biuret reaction by the dialysate of experimental urine number 24 bore a relation to that given by the fibrin hydrolysate solution similar to the relation found between the conjugated amino nitrogen concentrations of the 2 solutions. Of the conjugated amino acid nitrogen of normal human urine, ordinarily about half is extracted, after acidification, by ethyl acetate and is probably due largely to acyl amino acids,

[1] The term peptides will be used here for the dialyzable amino acid conjugates, although other nonprotein conjugates besides peptides may be included.

TABLE I

Plasma amino acid changes produced by the infusion of the fibrin hydrolysate

Five per cent solution in 5 per cent glucose, containing per liter 7.2 grams N, 1.98 grams free α-amino N, 3.44 grams bound α-amino N. Experiment 6, patient with duodenal ulcer; experiment 10, patient 3 days after hernioplasty; experiment 24, patient convalescent from diverticulitis, on low residue diet; experiment 14, normal person.

Experiment No.	Vol. infused	Time for infusion	α-amino N	Before infusion	Mid-infusion	End of infusion	1 hr. after	2 hrs. after	4 hrs. after	6 hrs. after
	ml.	*min.*		*mgm. per cent*						
6	1000	255	Free	4.80	6.02	6.49	4.26	3.94	4.18	
			In peptides	0.42	2.90	3.77	1.97	1.40	0.83	
10	875	165	Free	4.89		6.45	3.71	4.49		4.28
			In peptides	0.16		5.22	3.37	2.08		1.04
14	485	100	Free	4.64	6.15	5.93	4.36		4.68	
			In peptides	0.46	2.34	3.53	1.17		0.67	
24	1000	120	Free	5.15		8.47	6.00		4.35	
			In peptides	0.50		6.29	2.49		0.75	

TABLE II

Urinary loss of amino acids and peptides of a fibrin hydrolysate infused intravenously

Expt. No.*	Volume infused	α-amino N	Pretest period, fasting 12 hours	Infusion period and succeeding 6 hours	Extra loss: Net	Extra loss: Fraction of category infused
	ml.		*mgm. per hr.*	*mgm. per hr.*	*mgm.*	*per cent*
6	1000	Total	11.00	130	1070	20
		Free	3.44	22.2	164	8.3
		In peptides	7.56	108	900	26
10	875	Total	14.34	124.2	970	21
		Free	5.12	21.6	146	8.5
		In peptides	9.22	102.6	825	28
		Total extractable†	5.26	13.3	71	81
14	485	Total	13.01	105.3	542	21
		Free	4.30	20.2	93	9.7
		In peptides	8.71	85.1	448	27
24	1000	Total	2.66	149	1266	23
		Free	1.97	41.8	345	17
		In peptides	0.69	107	921	27
		Total extractable†	0.25	9.7	82	82
30**	730	Total	39.60	116	684	17.3
		Free	25.90	36	91	6.2
		In peptides	13.70	80	594	24

* See the notes to Table I.
† Extracted from acidified urine by 5 portions of ethyl acetate, 1.5 volumes each extraction.
** Hepatic cirrhosis; infusion in 165 minutes. See text.

mainly hippuric acid (3, 4), rather than peptides. The ethyl acetate-extractable conjugates of urine were increased by the infusion of the fibrin hydrolysate (Table II); the increases, however, represented only 9 per cent of the extra conjugated α-amino nitrogen excreted. About 1.8 per cent of the total α-amino nitrogen could be extracted from the hydrolysate itself under the same conditions. The additional extractable conjugates of the experimental urines may have arisen by preferential excretion of extractable peptides or by acylation of non-extractable peptides or amino acids.

To explore the possibility that impaired liver function might seriously handicap peptide utilization, 730 ml. of the fibrin hydrolysate solution were infused in 165 minutes into a 56-year-old male with

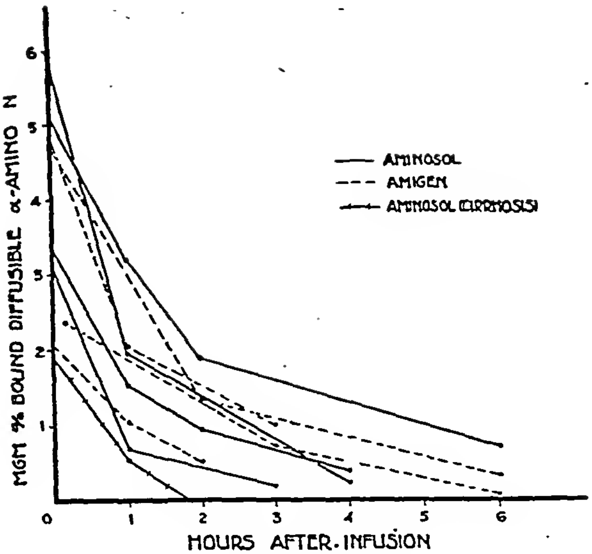

FIG. 1. REMOVAL FROM PLASMA OF THE PEPTIDES INTRODUCED BY THE INFUSION OF INCOMPLETE HYDROLYSATES OF FIBRIN AND CASEIN

The pre-experimental level of conjugated amino acid nitrogen has been deducted in each case. The term "Aminosol" refers to the experimental fibrin hydrolysate.

cirrhosis and ascites.[2] The patient yielded the following laboratory findings: bromsulfalein retention, 48 per cent after 1 hour (dose, 5 mgm. per Kilo); serum alkaline phosphatase, 9.6 King units; icterus index, 14; cephalin flocculation test, ++++; normal urea clearance. The pretest excretion of amino acid nitrogen (free and conjugated) was about a gram per day (Table II). This patient showed the smallest loss observed of the infused free amino acids and peptides (Table II). The rise in the plasma concentration of peptides at the end of the infusion was small, and these peptides were removed relatively quickly (Figure 1).

EXPERIMENTAL

The fibrin hydrolysate solution used was derived from a single lot and was supplied through the kindness of Dr. Douglas V. Frost of Abbott Laboratories. According to Dr. Frost the hydrolysis had been performed by acid at 100° for 6 hours, and the solution contained 7.2 grams of nitrogen per liter. Our analyses for this lot showed 1.98 grams of free α-amino nitrogen and 5.42 grams of total α-amino nitrogen (by acid hydrolysis) per liter, showing 64 per cent of the α-amino nitrogen to be bound. These analyses correspond quite well with those obtained by Frost for other lots.

Experimental details and analytical procedures were as described elsewhere (1).

DISCUSSION

The average sizes of the peptides of the 2 hydrolysates are similar, containing as an average, for Amigen (5), 3.4 and for the fibrin hydrolysate, 4.0 amino acid molecules per peptide molecules.[3] The difference in the availability of the peptides may be a result of differences in the 2 proteins, casein and fibrin, as to what amino acids are adjoining or may be a result of the different modes of hydrolysis, namely, by pancreatic enzymes and by acid. The first view that the difference is inherent in the proteins is supported by 2 conclusions of Frost, *et al.* (2), upon comparing the fibrin hydrolysate with a casein hydrolysate prepared in the same way by acid:

1. The superior biological value of the fibrin hydrolysate was not adequately explained by a higher content of essential amino acids.

2. Methionine in the state in which it is present in the casein hydrolysate appeared to be poorly utilized.

These observations suggest that the peptides of the hydrolysate of casein by acid, like those of the enzymatic hydrolysate, were not well utilized.

SUMMARY

Although our conclusion has been confirmed that peptides are less readily utilized than free amino acids when partial hydrolysates of protein are administered intravenously, the peptides of a fibrin hydrolysate were lost into the urine to a distinctly smaller extent than were the peptides of an enzymatic casein hydrolysate. The total amino acid losses of the 2 hydrolysates were similar. One may conclude that any partial hydrolysate of protein for intravenous nutrition offers the possibility of quantitatively or qualitatively serious losses of bound amino acids.

[2] This infusion was studied through the courtesy of Dr. Francis F. Harrison.

[3] The hydrolysis of fibrin has recently been extended to a degree of completeness similar to that of the casein hydrolysate. The term "Aminosol" is now applied to this preparation. The increased hydrolysis has intensified the difference in peptide utilization, urinary losses of peptides with the new preparation being, in 3 cases, only 16, 18, and 10 per cent—the total losses of amino acids, 8.4, 11, and 5.5 per cent.

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BIBLIOGRAPHY

1. Christensen, H. N., Lynch, E. L., and Powers, J. H., The conjugated, non-protein, amino acids of plasma. III. Peptidemia and hyperpeptiduria as a result of the intravenous administration of partially hydrolyzed casein (Amigen). J. Biol. Chem., 1946, 166, 649.
2. Frost, D. V., Heinsen, J., and Olsen, R. T., Partial acid hydrolysates of proteins. IV. Intravenous use of high levels of fibrin and casein hydrolysates in hypoproteinemic dogs. Arch. Biochem., 1946, 10, 215.
3. Henriques, V., and Sørensen, S. P. L., Über die quantitative Bestimmung der Aminosäuren, Polypeptide und der Hippursäure im Harne durch Formoltitration. Z. physiol. Chem., 1909, 63, 27.
4. Christensen, H. N., Cooper, P. F., Jr., Johnson, R. D., and Lynch, E. L. Glycine and alanine of body fluids; experimental modification. J. Biol. Chem., 1947, 168, 191.
5. Cox, W. M., Jr., and Mueller, A. J., The relative efficiency of different forms of intravenously administered nitrogen on nitrogen balance and amino acid excretion. J. Nutrition, 1946, 31, 581.

THE CONJUGATED, NON-PROTEIN, AMINO ACIDS OF PLASMA. V. A STUDY OF THE CLINICAL SIGNIFICANCE OF PEPTIDEMIA

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Peptidemia is a recurring concept of pathological chemistry which never has been either completely established or disproved. In a literature of about 100 papers only a few studies have been based upon methods which characterized the component measured as *amino acids in conjugated forms*[1]. The reported studies appear to indicate that intermediate protein degradation products frequently enter the blood in disease, so much so as to deprive their determination of diagnostic value.

Schweriner in 1920 (1) reported little if any peptides in mercuric chloride filtrates of normal blood but substantial amounts in neoplastic disease and in some types of liver disease. Blau (2) found elevated peptide nitrogen (1 to 8 mgm. per cent) in the blood in a number of diseases including 9 cases of hypertension. Becher and Herrman (3) found normal levels in carcinoma but high levels in severe renal insufficiency. Martens (4) reported concentrations of 8 to 13 mgm. per cent in severe hepatic and renal disease. Polonovski and Driesens (5) claimed that blood polypeptides were increased by as much as 4 times postoperatively, reaching a maximum 5 or 6 days after surgery. Hannaert and Wodon (6), Kalmykoff (7), Kotschneff (8), London and Kotschneff (9), and Martens (10) have reported evidence to show that ingested proteins were absorbed into the portal circulation partially as peptides and that the peptide content of the blood was modified upon flowing through the liver.

The principal ideas as to the clinical significance of peptidemia may be classified as follows:

1. That peptidemia is characteristic of neoplastic disease.

2. That peptidemia occurs after surgery or trauma or in certain types of shock, presumably because during increased protein catabolism, intermediate protein split-products may be released into the blood stream. This does not necessarily follow. Only slight increases of blood peptides were observed in proteose intoxication (11) and in histamine shock (12) although protein catabolism was strongly accelerated. "Peptide intoxication" as a humoral syndrome occurring after surgery, trauma or burns, proposed by Polonovski and Driessens (5), has been supported by several investigations. Postoperative peptidemia has been suggested as a precipitating factor in intravascular clotting (13).

3. That peptidemia occurs in hepatic disease, presumably because of the loss of a supposed hepatic function of clearing peptides from the blood.

4. That protein degradation products in the blood stream may be toxic and etiological agents in a number of diseases, including:

   (*a*) hypertension (2, 14),
   (*b*) toxemias of pregnancy (15).
   (*c*) intestinal strangulation.

Whipple, Rodenbaugh, and Kilgore (16) concluded that the toxic agent accumulating in closed intestinal loops was a primary heteroproteose. De Negri (17) reported that ligature of the mesentric arteries of dogs gave large increases of blood polypeptides. His analytical method was not specific, however. We have not considered

[1] Certain difficulties in terminology must be considered. The investigations reviewed here, and the present study as well, measured not *peptides* but *amino acid conjugates* which yield free amino acids upon hydrolysis. Although these conjugates usually have been supposed to be peptides, they may include proteins or such substances as hippuric acid. For simplicity we are using the inexact terms "peptides" and "peptide" nitrogen for these conjugates as determined by the procedures described here. Note that the ninhydrin method used by us yields all of the α-amino groups of a typical peptide as "peptide" nitrogen, none as free α-amino nitrogen, although one (the terminal) of these amino groups is not involved in a peptide bond. With the nitrous acid method, on the other hand, a dipeptide, for example, would yield one of its amino groups as free and the other as conjugated.

here the numerous other studies which have attempted to measure peptides by the difference in content of nitrogen (or of substances chromogenic with Folin's phenol reagent) in 2 types of filtrates of blood.

Even among those studies which have employed methods possessing some degree of specificity for conjugated amino acids, highly inconsistent results have been obtained for the normal levels of blood. These results have been tabulated elsewhere (18) together with a brief statement of the methods employed. Three factors have reduced largely the validity of such studies:

1. *Inadequate deproteinizing procedures.* Tungstic acid, picric acid and trichloroacetic acid, especially the latter in low concentration, all leave in solution *non-dialyzable* amino acid conjugates, presumably proteins (19). Dialysis of plasma must be very brief if proteolysis is to be avoided.

2. *Analysis of serum or defibrinated blood.* "Peptides" are formed in the course of clotting (19).

3. *Analysis of whole blood.* Erythrocyte glutathione contributes a major part of blood peptides. Apparent changes of blood peptides may arise from changes in hematocrit or from differences in the recovery of glutathione in blood filtrates (18).

In addition, the incomplete specificities of the analytical procedures, especially of the Folin amino acid method in its unmodified form, have probably contributed to the non-agreement.

Undetermined nitrogen in azotemia. In many cases of azotemia, notably "irreversible" azotemia following severe burns (20 to 22), a substantial part of the non-protein nitrogen has not been identified. The possibility frequently has been suggested that peptides might account for much of the unidentified nitrogen.

In brief, there is a wealth of ideas as to the pathological significance of peptidemia, but the experimental support is poor because of the inadequacy of the chemical procedures used. We have investigated the question employing the manometric ninhydrin procedure for amino acids, and using for the removal of proteins a combination of chemical deproteinization and dialysis (19). Such elevations of plasma "peptides" as we observed in a varied group of diseases were by no means as large as those previously reported, and were not sufficiently remarkable to give the determination diagnostic value, nor to contribute largely to the undetermined non-protein nitrogen of plasma.

EXPERIMENTAL

Plasma from heparinized venous blood (from fasting subjects except as indicated) was at once diluted with 4 volumes of redistilled water, and 5 volumes of dilute tungstic acid (23) or of 5 per cent trichloroacetic acid were added slowly. After 30 minutes the suspensions were centrifuged and the supernatant solution filtered. Free α-amino nitrogen was determined promptly upon 6 to 8-ml. aliquots of the tungstic acid filtrates at pH 2.5, or upon picric acid filtrates, by the manometric ninhydrin procedure (24). The result was corrected for the urea found present, except when a high urea concentration was anticipated, in which case urea was removed by urease.

A 30- to 50-ml. aliquot of the tungstic acid filtrate was dialyzed in a ¾-inch cellophane tube against a single measured portion (60 to 80 ml.) of redistilled water for 24 hours at 5° C. on a slowly rotating wheel. Trichloroacetic acid filtrates were first freed of trichloroacetic acid by ether extraction (18), concentrated in vacuum, made to 25 ml. and a 24-ml. aliquot likewise dialyzed. A maximal aliquot of the dialysate was concentrated and hydrolyzed in a sealed tube at 110° C. for 24 hours in 4N hydrochloric acid. The hydrolysate was taken to dryness *in vacuo*, remoistened and dried again, 10 ml. of water added, and 3-ml. aliquots of the resultant solution were analyzed for α-amino nitrogen at pH 2.5. The difference in the 2 analyses (total diffusible α-amino nitrogen minus free α-amino nitrogen) is the *conjugated diffusible* α-amino nitrogen, due to peptides and other amino acid conjugates. It is for this category that the term *"peptide" nitrogen* is used here, with the qualifications already noted.[1]

RESULTS

Normal concentrations. Twenty-one tungstic acid filtrates of plasma from persons in the postabsorptive state contained an average of 0.9 mgm. per cent of conjugated α-amino nitrogen (18). Such filtrates, however, contained from 0.2 to 0.5 mgm. per cent of conjugated α-amino nitrogen which was not dialyzable, this presumably being due to proteins (18). Six filtrates studied consecutively in June 1946 (19) contained no measurable dialyzable conjugates ("peptides"). Fourteen samples analyzed subsequently contained an average of 0.51 mgm. per cent (S.D. = 0.07; range 0.08 to 0.89 mgm. per cent) of "peptide" nitrogen. One may conclude that "peptides" are usually but not invariably demonstrable. Trichloroacetic acid filtrates generally showed slightly

higher concentrations of these "peptides" (range, from 0.0 to 1.5 mgm. per cent "peptide" nitrogen). Most of the difference, however, in the total nitrogen content of these 2 types of filtrates is due to protein.

TABLE I

Effect of the ingestion of milk or casein upon plasma amino acids

All filtrates prepared by tungstic acid. Dialysates of these filtrates were analyzed in experiments 6 to 10. All normal subjects except experiment 6.

Expt.	Meal	α-amino nitrogen	
		Free	"Peptide"
		mgm. per cent	
1	1 quart whole milk		
	postabsorptive	5.06	0.70
	150 min. after	5.54	−0.02
2	1 quart skim milk		
	postabsorptive	4.12	0.26
	60 min. after	4.07	0.16
	120 min. after	4.39	0.08
	200 min. after	4.98	0.03
3	1 quart skim milk		
	postabsorptive	3.97	1.70
	123 min. after	4.88	1.19
	180 min. after	4.86	0.74
4	400 ml. skim milk		
	postabsorptive	4.14	0.58
	113 min. after	4.31	0.25
	175 min. after	4.52	0.38
5	1 quart skim milk		
	postabsorptive	4.14	0.49
	120 min. after	4.70	0.12
6*	12 grams protein as cottage cheese		
	postabsorptive	3.45	0.51
	120 min. after	6.26	0.38
	180 min. after	5.98	0.14
7	30 grams casein		
	postabsorptive	4.33	0.55
	170 min. after	4.97	0.11
8	35 grams casein		
	postabsorptive	4.34	0.48
	150 min. after	5.84	0.48
	210 min. after	4.87	0.09
9	35 grams casein		
	postabsorptive	5.11	0.46
	180 min. after	6.30	0.64
10	35 grams casein		
	postabsorptive	5.23	0.61
	165 min. after	6.83	0.27
	225 min. after	5.73	0.44

* Child, age 3.3 years, weight 26 pounds, cystic fibrosis of pancreas. When the test meal was 18 grams of gelatin the plasma amino acid N did not rise above 4.7 mgm. per cent.

As to the nature of these "peptides," glycine analyses indicated that hippuric acid, glutathione or serylglycylglycine made no large contribution. From acidified tungstic acid filtrates only small fractions of the conjugates were extracted by ethyl acetate.

Effect of protein ingestion. The peripheral venous plasma did not contain increased quantities of "peptides" after the ingestion of 100 grams of gelatin (25) or of 35 grams of egg white protein. The ingestion of a quart of milk, or of 35 grams of casein, led not to an increase but to a *decrease* in the "peptides" of plasma (Table I). This strange effect has been obtained 9 times in 10 experiments. This effect was not produced, in control experiments, by: (*a*) continued fasting, (*b*) the ingestion of 35 grams of starch, (*c*) drinking 1.5 liters of water in the course of an hour. No explanation of the effect of casein ingestion will be attempted.

Do surgical procedures lead to peptidemia? A preliminary study upon a patient exposed to very extensive abdominal surgery (dismantling of gastrojejunostomy, excision of marginal ulcer, hemicolectomy, transverse colostomy, enterostomy) gave the following values for "peptide" nitrogen in plasma samples taken 42, 69, and 110 hours after surgery:

(*a*) Undialyzed tungstic acid filtrate, 0.25, 0.34, 0.8 mgm. per cent.

(*b*) Undialyzed trichloroacetic acid filtrate, 0.84, 0.80, 2.0 mgm. per cent.

Detailed study (Table II) of a group of patients exposed to various major surgical procedures failed to show any definite effect upon the "peptides" of plasma (although the free amino acids were always reduced [26]) or upon the urinary excretion of free or conjugated amino acids. The preoperative and postoperative concentrations of plasma "peptides" were somewhat higher than the average for normal subjects, but not outside the normal range. The increased protein catabolism which occurs postoperatively apparently does not result in the appearance of increased amounts of intermediate degradation products in the plasma.

Peptidemia in various diseases. Table III summarizes our observations upon patients with various diseases. Peptidemia did not appear to be characteristic of neoplastic diseases, hepatic dis-

TABLE II

Effect of surgery upon free and conjugated amino acids of plasma and urine

Surgical procedure	α-amino N	Plasma Interval after surgery							Urine	
		Preopt.	Immed.	1 day	2 days	4 days	7 days	10 days	Preopt.	Postop.*
		mgm. per cent							*mgm. per hour*	
Carcinoma of sigmoid colon: Resection, sigmoidoscopy, exteriorization of sigmoid	Free	4.96	3.93	4.28	3.84	4.50	4.72	4.34	7.27	9.50
	Peptides	1.68			1.40	1.14	1.40		5.90	5.80
	Peptides†	0.75	0.76	0.66	0.98	0.87	0.25	0.90		
Open reduction of comminuted fracture of tibia with application of bone plate	Free	3.99	2.93	2.79	4.08	3.76	3.60		3.17	5.09
	Peptides	0.41	0.77	0.47	0.73	1.02	0.44		4.10	7.10
	Peptides†	0.55	0.68	0.80	0.79	0.87				
Open reduction of fracture of radius and ulna with application of bone plate	Free	4.18	2.98	4.08	3.60	4.20			4.81	5.83
	Peptides	1.17	0.96	0.89	0.55	0.81			6.60	5.66
Exploration of kidney, ureterotomy with removal of stone	Free	3.92		3.83	3.41	4.47	4.07		4.98	4.36
	Peptides	1.06		0.50	0.74	0.79	0.58		4.73	4.11
Peritonitis with obstructive symptoms; exploration	Free	3.3	3.28							
	Peptides	1.3	1.25							
Carcinoma of rectum; bilateral radical dissection of inguinal lymph nodes	Free	4.69	2.42	2.63	2.93	3.44	3.78	3.87**	6.86	6.94
	Peptides	0.70	0.53	0.67	0.69	0.79	0.88	0.40	11.80	9.40

* All urine excreted during a 72- to 96-hour period beginning just before the operation. The preoperative urines were collected during the preceding 12 hours.

† Tungstic acid filtrates of plasma. All other values for "peptide" N of plasma were determined upon 2.5 per cent trichloroacetic acid filtrates.

** Fourteenth day after surgery, free α-amino N, 4.03 mgm. per cent; "peptide" N, 0.39 mgm. per cent.

ease, even if severe, of venous thrombosis, of severe burns without azotemia, or of a number of other conditions. Somewhat elevated values appeared to occur in the nephrotic syndrome, and also in some cases of azotemia, but no level above 2.5 mgm. per cent of "peptide" nitrogen has been observed. While one cannot conclude that the rather small elevations observed in disease are without physiological significance, no indication of diagnostic value of the analysis has been obtained, nor do peptides and other conjugates appear to make any major contribution to the undetermined non-protein nitrogen of plasma.

Subjects showing the higher concentrations of "peptides" in the plasma did not regularly excrete larger quantities in the urine. On the other hand whenever the plasma peptide nitrogen is elevated by a few mgm. per cent by the intravenous injection of peptides (in the form of incomplete hydrolysates of protein) there is a massive peptiduria (27, 28). A dissimilarity is apparent in the nature of the "peptides" of plasma in the two situations.

Other body fluids. Several cerebrospinal fluid samples (by lumbar puncture) contained from 0.2 to 0.8 mgm. per cent of "peptide" nitrogen (Table IV). Tungstic acid, trichloroacetic acid and picric acid all left in solution a large fraction (15 to 50 per cent) of the protein of normal cerebrospinal fluid. Seminal plasma was unusually rich in free amino acids and in peptides, probably because of the presence of strong proteolytic activity, which led to the release of nearly 100 mgm. per cent of free amino acid nitrogen in 12 hours at 20°. Pathological accumulations of chest fluid, ascitic fluid, and synovial fluids appeared to contain "peptide" nitrogen in the range of 1 to 3 mgm. per cent.

SUMMARY

1. Factors which largely vitiate the validity of former determinations of blood peptides have been considered. The conjugated non-protein, amino acids (peptides plus other conjugates) of the plasma of normal persons and patients have been examined by procedures designed to avoid such factors.

TABLE III

"Peptides" of plasma in various diseases

TCA = 2.5 per cent trichloroacetic acid

No.	Diagnosis	α-amino nitrogen		Type of filtrate
		Free	"Peptide"	
		mgm. per cent		
32540	Early carcinoma of cervix; x-ray therapy	3.16	0.88	TCA
16610	Carcinoma of the prostate	3.57	0.52	TCA
31194	Carcinoma of the pancreas; jaundice	3.76	0.10	TCA
24460	Adenocarcinoma of the common bile duct; metastases to liver	3.70	0.9	Tungstic
			1.7	TCA
31905	Adenocarcinoma of liver	4.79	0.92	Tungstic
			1.22	TCA
32621	Carcinoma of unknown primary site—pressure on common bile duct	3.7	0.7	TCA
31956	Infectious hepatitis	4.62	0.19	Tungstic
			0.40	TCA
17291	Infectious hepatitis	5.08	1.24	TCA
32401	Cirrhosis with ascites	4.22	0.67	TCA
32314	Infectious hepatitis	4.11	1.11	TCA
27094	Infectious hepatitis	4.67	0.30	Tungstic
			0.83	TCA
29862	Metastatic carcinoma of liver; cholemia	5.32	0.73	Tungstic*
27698	Obstructive jaundice; serum proteins 4.1 per cent	5.56	0.52	Tungstic*
17708	Diabetic acidosis; serum CO_2 7.6 mM. per liter	8.93	2.40	Tungstic*
	7 hours later; serum CO_2 26.1 mM. per liter	3.50	2.27	Tungstic*
31242	Diabetic acidosis; serum CO_2 6.1 mM. per liter	8.20	0.26	Tungstic
	12 hours later; serum CO_2 26.2 mM. per liter	2.80	1.0	TCA
31935	Addison's disease, in relapse during withdrawal of steroid therapy;	5.0	0.7	Tungstic
	diabetes mellitus		1.6	TCA
32717	Pneumonia and empyema	3.16	0.01	TCA
14886	Bleeding duodenal ulcer	3.85	0.74	TCA
31265	Thrombosis of deep femoral veins; after venous ligation	3.56	0.83	TCA
31770	Thrombosis of saphenous vein; 24 hours after ligation (recurrent)	3.40	1.27	TCA
28777	Thrombosis of superficial femorals; after ligation	3.49	0.97	TCA
31488	Burns covering 28 per cent of surface. No azotemia	2.37	0.52	Ultrafiltrate
32268	Burns, 40 per cent of surface. No azotemia. After 1 day	2.93	0.65	Tungstic
			1.86	TCA
	After 2 days	3.72	0.72	Tungstic
			2.04	TCA
	After 3 days	3.18	1.67	Tungstic
19421	2.3 hours postpartum	2.81	0.66	TCA
10253	1 hour postpartum	2.83	0.49	TCA
23559	Toxemia of pregnancy	3.71	1.02	TCA
32646	Uremia; hypertensive cardiovascular disease	5.74	1.05	TCA
32734	Polycystic kidneys (no azotemia)	3.97	2.37	TCA
28161	Terminal nephritis with nephrotic component, age 13	3.40	1.58	Tungstic*
31276	Nephrosis, age 17 months	3.4	0.7	Tungstic
31276	Nephrosis, age 19 months	3.4	1.43	TCA
313068	Nephrosis,† age 3.3 years	3.53	1.58	TCA
309517	Nephrosis,† age 3.3 years	3.86	2.12	TCA
H9	Nephrosis,† age 4 years	4.94	1.00	TCA
29376	Nephrosis, age 6 years	3.35	1.25	Tungstic*
30627	Nephrotic stage of nephritis, anasarca, 58 years	4.09	0.66	Tungstic*
B11	Nephrotic stage of nephritis, child	4.06	0.78	TCA
C11	Nephrotic stage of nephritis, child	3.65	1.42	TCA
R21	Renal rickets, amyotonia,** age 2.5 years	3.25	1.0	Tungstic
17880	Cystic fibrosis of the pancreas	3.45	0.51	Tungstic*
33596	Toxemia of pregnancy	3.35	0.16	TCA
14666	Toxemia of pregnancy	3.61	0.48	TCA
33581	Rheumatoid arthritis, hypervitaminosis D, calcinosis, azotemia	4.22	0.53	TCA

* Analyses upon undialyzed filtrates; hence, some protein presumably is included.
† Sample supplied by Dr. Gretchen Hutchens, Children's Hospital, Boston.
** Sample supplied by Dr. Nathan Talbot, Massachusetts General Hospital, Boston. The urinary excretion of free α-amino acid nitrogen was 150 mgm. per day; of glycine nitrogen, 31 mgm. per day.

TABLE IV

Amino acids and "peptides" of some body fluids

No.	Fluid; diagnosis	α-amino nitrogen		Type of filtrate
		Free	"Peptide"	
L 27	Cerebrospinal fluid, normal	1.41	0.8	TCA
J 55	Cerebrospinal fluid, hydrocephalus, ventricular tap	1.04	0.10	TCA
K 54	Cerebrospinal fluid, normal	1.16	0.48	TCA
K 50	Cerebrospinal fluid, meningococcal meningitis	1.06	0.74	TCA
M 8	Cerebrospinal fluid, normal	1.80	0.20	Dialysate
E 72	Seminal plasma	15.8*	14.0*	Picric
	Same, after 12 hours at 20°	103.6		Picric
N 21	Chest fluid, measles, pleurisy	1.52		Picric
K 32	Chest fluid, constrictive pericarditis 7/9	4.73	1.31	TCA
K 32	Chest fluid, constrictive pericarditis 7/19	3.34	2.68	TCA
K 43	Ascitic fluid, sec. carcinoma of peritoneum	3.83	0.91	TCA
J 33	Synovial fluid, from knee, rheumatoid arthritis	4.43	1.10	TCA

* Three other experiments gave similar findings. Free glycine N 0.62 mgm. per cent; alanine N 1.4 mgm. per cent; little, if any, glutamine.

2. Tungstic acid filtrates of the plasma of normal fasting persons contained usually less than 1 mgm. per cent of conjugated, non-protein, amino acid nitrogen, and in some cases the concentration was too small to be measured. Protein ingestion did not increase the concentration of conjugates; in fact, milk or casein produced decreases.

3. Surgical procedures failed to produce increases of the "peptides" of plasma, nor were significant changes produced in the urinary excretion of free or conjugated amino acids.

4. While the plasma of patients with a variety of diseases contained as an average somewhat more amino acid conjugates than the plasma of normal persons most of the values were not outside the range encountered in normal persons. Elevated values did not appear to be characteristic of neoplastic disease, hepatic disease, or venous thrombosis. Somewhat elevated values were observed in nephrosis, but no value above 2.5 mgm. per cent of "peptide" nitrogen has been observed.

5. The free and conjugated amino acids of a few other body fluids have been reported.

BIBLIOGRAPHY

1. Schweriner, F., Der anteil der Polypeptide und aminosauren am reststickstoff des Blutes. Z. exp. Path. u. Therap., 1920, 21, 129.
2. Blau, N. F., The amino acid nitrogen of the blood. III. A study of the occurrence of peptide nitrogen in the blood. J. Biol. Chem., 1923, 56, 873.
3. Becher, E., and Herrmann, E., Der freie und gebundene Aminostickstoff im enteiweissten Blut und Gewebe unter normalen und pathologischen Verhältnissen; III. Befunde im blut bei Krankheiten, insbesondere bei Leberkrankheiten. IV. der freie und gebundene Amino-N im enteiweissten Blut bei essentieller Hypertonie und Nierenkrankheiten. Deutsches Arch. f. klin. Med., 1932, 173, 1, 23.
4. Martens, R., Contribution à l'étude de l'azote peptidique dans le sang. Bull. Soc. chim. biol., 1928, 10, 1336.
5. Polonovski, M., and Driessens, J., L'hyperpolypeptidémie et l'azotémie postopératoires in chirurgie générale. Compt. rend. Soc. de biol., 1931, 107, 324.
6. Hannaert, L., and Wodon, R., Contribution a l'étude de hemoclasie digestive. Compt. rend soc. Biol., 1923, 88, 636.
7. Kalmykoff, M. P., Abbau im darm und aufbau in der leber bei eiweissresorption. Arch. f. d. ges. Physiol., 1924, 205, 493.
8. Kotschneff, N., Weitere Untersuchungen über das Verhalten verschiedener Eiweissabbauprodukte im Intermediargebeit nach Versuchen an angiostomierten Hunden. Arch. f. d. ges. Physiol., 1928, 218, 635.
9. London, E. S., and Kotschneff, N. P., In welcher form wird nahrungseiweiss resorbiert? Z. Physiol. Chem., 1934, 228, 235.
10. Martens, R., Rôle de la paroi intestinale et du foie lors de la résorption des produits de digestion des protéides. Bull. Soc. chim. biol., 1931, 13, 1187.
11. Whipple, G. H., and Van Slyke, D. D., Proteose intoxications and injury of body protein. III. Toxic protein catabolism and its influence upon the non-protein nitrogen partition of the blood. J. Exper. Med., 1918, 28, 213.
12. Hiller, A., The effects of histamine on protein catabolism. J. Biol. Chem., 1926, 68, 847.
13. Ochsner, A., Intravenous clotting. Surgery, 1945, 17, 240.
14. Jackson, H., Jr., Sherwood, D. W., and Moore, O. J., The blood peptide nitrogen in arterial hypertension. J. Biol. Chem., 1927, 74, 231.
15. Schlossmann, H., Der gehalt des blutes in aminosauren und polypetiden in schwangerschaft, geburt und wochenbett mit besonderer berucksichtigung der schwangerschaft toxamien. Z. ges. exptl. med., 1925, 47, 487.
16. Whipple, G. H., Rodenbaugh, F. H., and Kilgore, A. R., Intestinal obstruction; proteose intoxication. J. Exper. Med., 1916, 23, 123.
17. De Negri, L., Dei polypeptidi e di altre frazioni azotate nell'occlusione e necrosi dell'intestino. Riv. patol. sper., 1938, 21, 15.
18. Christensen, H. N., and Lynch, E. L., The conjugated, non-protein amino acids of plasma. I. Postabsorptive concentrations of human plasma, serum and erythrocytes. J. Biol. Chem., 1946, 163, 741.

19. Christensen, H. N., and Lynch, E. L., The conjugated non-protein amino acids of plasma. II. A study of deproteinizing techniques. J. Biol. Chem., 1946, 166, 87.
20. Taylor, F. H. L., Levenson, S. M., Davidson, C. S., and Adams, M. A., Abnormal nitrogen metabolism in patients with thermal burns. New England J. Med., 1943, 229, 855.
21. Walker, J., Jr., A study of the azotemia observed after severe burns. Surgery, 1946, 19, 825.
22. Rosenthal, O., and McCarthy, M. D., Post-burn azotemia, its characteristics and relationship to the severity of thermal injury. Am. J. Physiol., 1947, 148, 365.
23. Van Slyke, D. D., and Hawkins, J. A., A gasometric method for the determination of reducing sugars, and its application to analysis of blood and urine. J. Biol. Chem., 1928, 79, 739.
24. Hamilton, P. B., and Van Slyke, D. D., The gasometric determination of free amino acids in blood filtrates by the ninhydrin-carbon dioxide method. J. Biol. Chem., 1943, 150, 231.
25. Christensen, H. N., Cooper, P. F., Jr., Johnson, R. D., and Lynch, E. L., Glycine and alanine concentrations of body fluids; experimental modification. J. Biol. Chem., 1947, 168, 191.
26. Man, E. B., Bettcher, P. G., Cameron, C. M., and Peters, J. P., Plasma α-amino acid nitrogen and serum lipids of surgical patients. J. Clin. Invest., 1946, 25, 701.
27. Christensen, H. N., Lynch, E. L., and Powers, J. H., The conjugated, non-protein, amino acids of plasma. III. Peptidemia and hyperpeptiduria as a result of the intravenous administration of partially hydrolyzed casein (amigen). J. Biol. Chem., 1946, 166, 649.
28. Christensen, H. N., Lynch, E. L., Decker, D. G., and Powers, J. H., The conjugated, non-protein, amino acids of plasma. IV. A difference in the utilization of the peptides of hydrolysates of fibrin and casein. J. Clin. Invest., 1947, 26, 849.

STUDIES OF BLOOD VOLUME IN THE TETRALOGY OF FALLOT AND IN OTHER TYPES OF CONGENITAL HEART DISEASE[1]

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The recent introduction by Blalock (1) of an operative procedure for the successful treatment of the tetralogy of Fallot has focused considerable attention on the various manifestations of this condition. It has also indicated the need of more information to aid in the diagnosis and prognosis, as well as in the preoperative and postoperative treatment of the patients with this disease. The present study is concerned particularly with the measurement of blood volume, hemoglobin, and plasma protein in 7 cases of tetralogy of Fallot, 4 cases of isolated septal defect, and 1 case each of Eisenmenger complex, isolated pulmonic stenosis, coarctation of the aorta, congenital mitral stenosis, and patent ductus arteriosus.

Fallot (2), in 1888, described the complex of combined congenital cardiac defects of pulmonic stenosis, right ventricular hypertrophy, patent interventricular septal defects, and dextroposition of the aorta which is commonly referred to as the "Tetralogy of Fallot." It is a common congenital defect of the heart. It constitutes 77 per cent of the 110 cases of pulmonary stenosis and 66 per cent of 40 cases of pulmonary atresia in Abbott's (3) series of 1,000 cases. The Eisenmenger complex is much less common. It differs from Fallot's tetralogy by having no pulmonary stenosis or atresia. The prognosis is somewhat better, the average life span being 16 years compared to 12.5 years in Fallot's type (4). The oldest age of survival recorded in either type is 60 years (5).

The principal extracardiac pathology of Fallot's tetralogy is a generalized vascular distention, capillary hyperplasia (3), and increased tortuosity. This is accompanied by polycythemia and increased blood viscosity. Blood arteriovenous-oxygen difference is increased, and its per cent oxygen saturation is decreased. Cyanosis, usually present at rest, is increased on exertion, when dyspnea also appears.

Although the elevated values of hemoglobin and hematocrit in the tetralogy of Fallot suggest an increased blood volume, measurements of blood volume have been recorded in relatively few cases of this disease. Meyer (6) measured the volume in 1 case using congo red, as did Blumenfeldt and Wollheim (7) using trypan blue. Both authors reported extremely low values for plasma volumes. Weber and Dorner (8), using the carbon monoxide method, obtained a value of 131 ml. per kgm. or a total blood volume of 6,040 ml. in a patient with cyanotic congenital heart disease (morbus caeruleus) as compared with the value of 77 ml. per kgm. in normal individuals measured by their method. Hallock (9) used the T–1824 blue dye in studying 2 cases. He found low plasma volumes (29.9 and 29.3 ml. per kgm.) and high cell volumes (144 and 77 ml. per kgm.).

METHODS AND PROCEDURE

Considerable care was devoted to the standardization of the procedures and methods used. All patients were studied under basal conditions. Breakfast was omitted, and the patients rested quietly in the recumbent position for at least 10 minutes before the determinations were made. Blood was drawn from an antecubital vein into syringes coated with a thin film of heparin. Total circulating plasma volume was determined by the method of Gregersen (10) using the Klett-Summerson photoelectric colorimeter. The amount of heparin which was used did not interfere with accurate determination of the plasma volume. To rule out the question of possible inadequate mixing because of slowed circulation, several successive samples were taken at 5-minute intervals after the usual 10-minute sample. In a number of cases, simultaneous samples were taken from the dorsal foot vein and an antecubital vein. All of our cases showed complete mixing in 10 minutes, and there was no evidence that the disappearance curve of the dye differed from that given for "normal" individuals. All adults were injected with 5

[1] One of series of studies being conducted under a grant from the Research and Development Division, Office of the Surgeon General, United States Army.

ml. of dye. Children were given a smaller amount of dye roughly proportional to their normal weight.

Hematocrit values were obtained by centrifuging blood in Wintrobe tubes. Since spinning for 30 minutes at 3,000 revolutions per minute gave the same results as spinning for an hour, the former interval was used in all of the experiments. Hematocrit determinations were made on the samples of blood taken before and after the injection of the dye. This served as a check on possible fluid shifts during the 10-minute intervening period.

Red cell volumes were calculated from the hematocrit values. The absolute accuracy of this procedure has been questioned by some (11) and affirmed by others (12). The suggested discrepancy between the true red cell volume and that calculated from the hematocrit, if present, would tend to make our absolute values uniformly too high by a maximum of about 25 per cent . It would have less bearing on the relationship of the actual values to standard values obtained by the same procedure.

Hemoglobin was determined as oxyhemoglobin, using the photoelectric colorimetric method of Evelyn (13). Oxygen capacity determination by the Van Slyke-Neill method furnished the necessary constants for calculation of the actual values. Specific gravity of the plasma was determined by the falling-drop method (14), and plasma protein levels were calculated from these values by the formula of Weech, Reeves, and Goettsch (15). All readings were made as soon as possible after the blood was drawn. A series of determinations made at intervals on 9 "normal" subjects over a period of 4 months showed fluctuations of not more than 10 per cent. Changes of greater magnitude have therefore been considered as significant.

The values given by Gregersen (10) were used to assign standard levels in the adult patients for blood volume, plasma volume, red cell volume, total plasma protein, and total circulating hemoglobin. These values were assembled by Gregersen from a large series and agree in

TABLE I

Tetralogy of Fallot

Patient number	Age	Obs. wt.	Date 1946	Hct.	Hb.	Hb.	Plasma protein	Plasma protein	Plasma volume	Red cell volume	Blood volume	Comments
		kgm.		*per cent*	*grams per cent*	*grams per kgm.*	*grams per cent*	*grams per kgm.*	*ml. per kgm.*			
204	19 mos.	6.8	7-9	60	14.3	28.7	6.09	4.9	80.3	120.5	201.7	12 pounds under average weight
208	22 yrs.	57.6	1-31	76	19.7	51.5	8.05	5.1	64.1	195.6	259.7	
		57.6	2-12	70	20.7	40.0	6.85	4.0	57.8	135.1	193.1	
		54.9	3-12	68	20.2	32.0	6.85	3.3	48.5	109.8	158.3	
		57.6	3-26	59	17.2	18.9	5.88	2.6	44.8	64.6	109.5	
		57.6	5-9	64	16.6	24.9	6.99	3.3	54.0	95.9	149.9	
		58.1	7-18	66	20	25.3	7.06	3.0	43.0	83.5	126.5	Blalock procedure 7-30-46
		57.6	7-29	70	20.3	23.2	6.68	2.3	34.3	80.0	114.3	Died 8-1-46
207	20 yrs.	49.0	3-28	70	19.8	33.6	7.34	4.0	54.0	126.0	180.0	27 pounds under usual weight Died 4-9-46. Autopsy
205	4 yrs.	15.9	4-30	75	19.5	45.8	7.69	4.0	51.4	154.1	205.7	
		18.1	7-29	70	20.5	21.9	6.99	2.4	32.0	74.9	106.9	
206	44 yrs.	46.3	5-7	66	19.6	31.0	5.81	3.1	54.0	104.9	158.9	18 pounds under usual weight Neoplastic cachexia developing symptom from carcinoma of stomach
		44.5	6-18	65	19.6	33.5	5.39	3.2	59.8	111.0	170.8	
		44.5	7-5	63	18.5	24.5	6.37	3.1	49.0	83.4	123.4	Died 7-31-46. Autopsy
212	6 yrs.	13.7	7-29	81	20.5	63.1	6.41	3.7	58.4	294.3	307.7	15 pounds under average weight with marked mental deficiency
		13.7	8-15	81	21.8	93.4	6.16	5.0	81.4	346.9	428.3	
211	6 yrs.	14.1	6-20	73	21.4	44.9	6.65	3.8	56.7	153.2	209.9	15 pounds under average weight
		14.1	6-29	74	20.1	52.6	7.30	5.0	68.1	193.6	261.7	Blalock procedure 7-20-46
		14.1	8-7	62	16.2	24.8	7.09	4.1	58.2	94.9	153.2	
		14.1	8-15	59	17.3	17.8	4.80	2.0	42.1	60.4	102.8	
		14.1	9-10	61	19.2	22.1	6.9	3.1	44.8	70.1	115.0	

general with our findings and with those of other series (16). Standard values for blood, plasma, and red cell volumes in the children were calculated from the data given by Brines, Gibson, and Kunkel (17). Wintrobe's standard values were used for hemoglobin and hematocrit (18). Since all of our children were over 18 months old, their standard plasma protein level was assumed to be similar to that of the adult, *i.e.*, 6.5 grams per cent.

Diagnosis of tetralogy of Fallot was made principally on a basis of: (*1*) a history of cyanosis since, or shortly after, birth; (*2*) physical findings of a pulmonic stenosis-type murmur, cyanosis, finger and toe clubbing, and watchcrystal nail changes; and (*3*) X-ray findings of decreased conus pulmonus prominence. Retarded physical development was prominent in all of the children studied, and retarded mental development was observed in 1 case. Associated congenital defects were noted in 1 case. Rest cyanosis was present to a greater or lesser degree in all cases, and its presence in the absence of dyspnea and tachycardia was striking. Congestive failure was found in only 1 case (No. 206), and only 1 case (No. 212) showed marked cardiac enlargement. Right axis deviation was consistently found electrocardiographically. Circulation times, intracardiac diodrast studies, and oxygen analyses were done on some of the cases and showed markedly reduced arm-to-tongue or -face circulation time, right ventricle retention and right-to-left heart shunt, and decreased arterial oxygen saturation and increased arteriovenous oxygen difference. The aortic arch was on the left side in all cases. Two of the cases (Nos. 206 and 207) came to autopsy and presented the typical picture of the tetralogy of Fallot.

Case No. 210 was classified as an example of the Eisenmenger complex mainly on the basis of a prominent conus, lack of aortic prominence, and lack of a murmur typical of pulmonic stenosis. Since it was not feasible to employ venous catheterization, the possibility of an isolated inter-arterial septal defect was not definitely excluded. This patient manifested cyanosis only on exertion. The patient with isolated pulmonic stenosis showed a markedly prolonged retention of diodrast in the right ventricle and no evidence of septal defect. The diagnosis of patent interventricular septal defect was based primarily on the history and character of the murmur.

RESULTS

A total of 22 sets of determinations was done in the 7 cases of tetralogy of Fallot, 2 sets of determinations each in the case of the Eisenmenger complex and in the case of coarctation of the aorta. Single sets of determinations only were obtained in the cases of septal defect, congenital mitral stenosis, patent ductus arteriosus, and in isolated pulmonic stenosis.

TABLE II

Miscellaneous Congenital Heart Disease

Case No.	Diagnosis	Age	Obs. wt.	Date 1946	Hct.	Hemoglobin	Hb.	Plasma protein	Plasma protein	Plasma volume	Cell volume	Blood volume	Comments
			kgm.		*per cent*	*grams per 100 ml.*	*grams per kgm.*	*grams per cent*	*grams per kgm.*	*ml. per kgm.*			
209	Isolated pulmonic stenosis	22 yrs.	57.7	5-21	62	18.0	20.9	6.40	2.8	44.1	72.0	115.9	
210	Eisenmenger complex	16 yrs.	47.6	5-31	49	14.9	12.6	7.65	3.86	50.4	48.4	98.8	
214	Coarctation of aorta	20 yrs.	47.2	6-6	44	13.1	11.1	7.27	3.43	47.4	37.2	84.5	
			47.2	6-20	38	11.6	9.4	6.68	3.37	50.4	39.0	81.4	
213	Patent ductus arteriosus	9 yrs.	20.0	7-27	38	13.3	14.5	7.44	5.95	79.8	48.8	128.5	Underweight No cardiac decomposition
215	Congenital mitral stenosis	22 mos.	10.4	8-13	37	11.0	19.0	6.68	7.31	109.0	63.9	172.7	In congestive failure
220	I.V. septal defect	5 yrs.	18.1	9-26	39	12.4	11.2	6.58	3.59	54.9	35.1	89.9	
221	I.V. septal defect	5 yrs.	16.3	9-24	28	8.7	7.0	7.34	4.23	57.8	22.5	80.2	
222	I.V. septal defect	5 yrs.	16.3	9-24	34	10.8	9.7	7.37	4.36	59.0	30.4	89.6	
223	I.V. septal defect	10 yrs.	27.2	10-1	36	11.8	4.9	7.52	3.12	41.7	23.4	65.1	

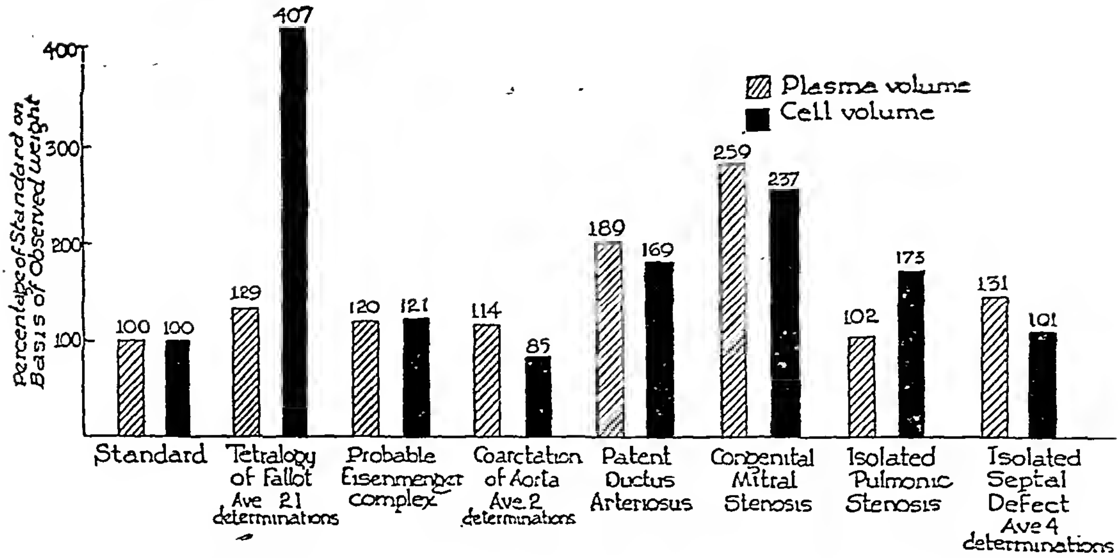

FIG. 1. COMPARISON OF TETRALOGY OF FALLOT WITH OTHER TYPES OF CONGENITAL HEART DISEASE

The results are given in detail in Tables I and II. A comparison of the plasma and red cell volumes in the various conditions is given in Figure 1. Our findings, contrary to those of previous investigators, indicate that the plasma volume is not significantly or consistently reduced in the tetralogy of Fallot.

The highest value for the total blood volume was 14,900 ml. (259 ml. per kgm.) found in the first determination in Case 208. To our knowledge, this is the largest blood volume reported in this disease, although Hallock (9) reported a case of polycythemia vera with a blood volume of over 18 liters. This case (No. 208) is of additional interest because of the dramatic response to bed rest, the blood volume dropping progressively from over 14,900 ml. to 6,300 ml. in a period of 2 months (Figure 2). This occurred without any

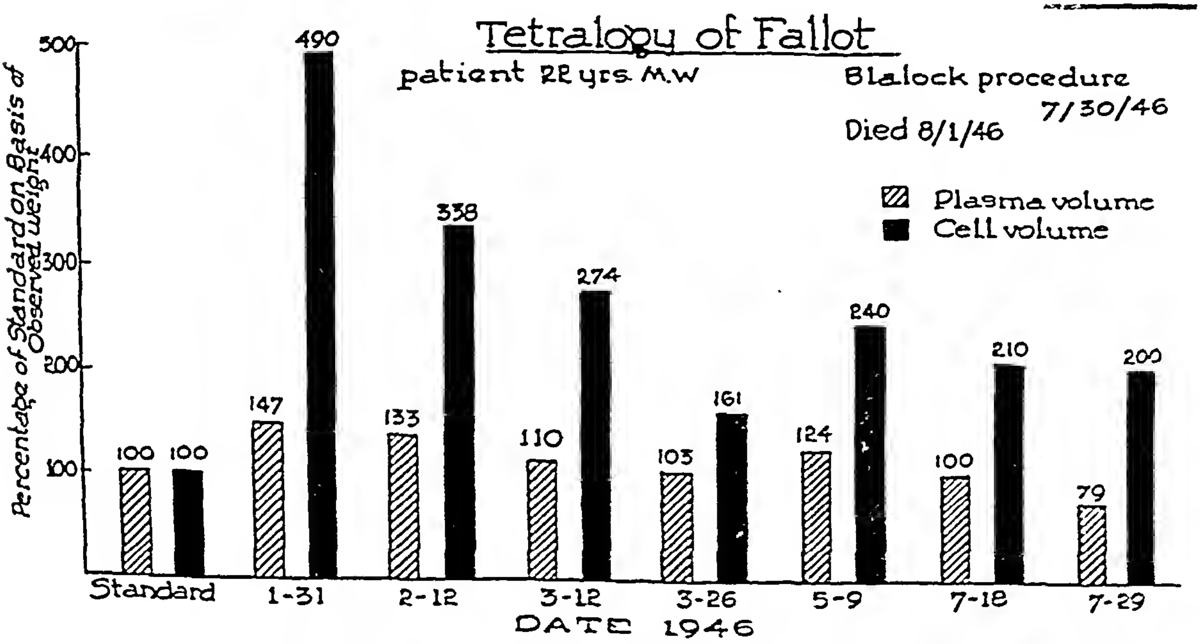

FIG. 2. SERIAL DETERMINATIONS OF PLASMA AND CELL VOLUMES IN A PATIENT 22 YEARS OF AGE (M. W.) WITH TETRALOGY OF FALLOT

hemorrhage and without clinical evidence of hemolysis. This decrease, while primarily due to a drop in the red cell volume, was accompanied by a drop in the plasma volume and total protein. This same tendency is also seen in Case 205, although the number of observations in this case is limited.

The highest total blood volume per kilogram was seen in Case 212. This child showed the most severe clinical picture of those surviving in the group. He manifested maximum cyanosis, exertional dyspnea, and cardiac enlargement. His initial blood volume was 4,215 ml. (307.7 ml. per kgm.), and a second determination some 2 weeks later showed a value of 5,268 ml. (428. 3 ml. per kgm.).

Initial values for plasma volume ranged from 51.4 to 80.3 ml. per kgm. These are 20 to 92 per cent above standard level as based on the patient's observed weights. Five of the patients were underweight at the time they were studied. We have found that in such individuals, plasma and cell volumes are more accurately predictable on the basis of optimal or usual weight. When the initial plasma volumes are calculated on this basis, the values in 4 of the 7 cases are almost exactly equal to the predicted or standard values. The remaining 3 cases (Nos. 204, 205, and 208) show values 111, 121, and 147 per cent, respectively, of the predicted levels. On the other hand, all of the patients showed strikingly increased cell volumes which varied from 104.9 to 294.3 ml. per kgm. These values are from 269 to 998 per cent of standard on an observed weight, and 220 to 639 per cent on an optimal or usual weight basis. Calculation of the total circulating hemoglobin revealed high values consistent with the elevated total cell volume. The initial values varied from 28.7 grams per kgm. to 63.1 grams per kgm. and represented levels which were from 242 to 764 per cent of the standard on an observed, and 208 to 527 per cent on an optimal or usual, weight basis.

Two of the cases of tetralogy (Nos. 208 and 211) were operated upon (see case reports). Case 208 survived only 3 days, while Case 211 has

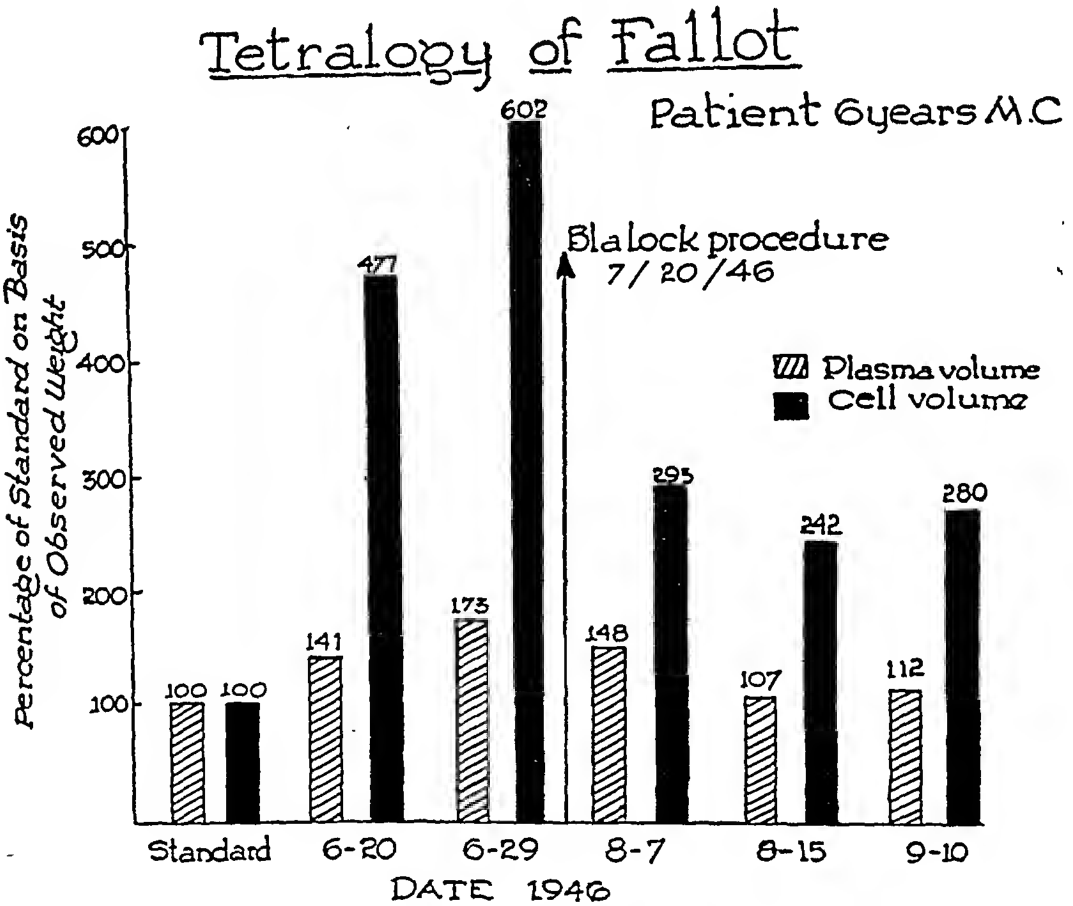

Fig. 3. Serial Determinations of Plasma and Cell Volumes in a Patient 6 Years of Age (M. C.) with Tetralogy of Fallot

shown definite improvement. Determinations made postoperatively in Case 211 showed a progressive decline in the total red cell volumes. The total blood volume 1 month after operation was only moderately increased over the standard level for the patient's observed weight but showed a slight rise following resumption of normal activity (Figure 3).

As in the tetralogy, the values for plasma volume in other types of congenital anomalies show variations which are near or slightly above predicted standard values, except for the case of patent ductus arteriosus (Case 213) and of congenital mitral stenosis (Case 215). In the latter cases, the values were 79.8 ml. per kgm. and 109.0 ml. per kgm., respectively, which are 189 and 259 per cent of standard on an observed, and 140 and 229 per cent of standard on an optimal, weight basis. Case 215, of congenital mitral stenosis, was in probable congestive failure preceding and at the time of the determinations.

In contrast to the findings in the tetralogy, however, only 3 cases showed high total red cell volumes. Again, the patent ductus arteriosus and congenital mitral stenosis show relatively high values (48.8 and 63.9 ml. per kgm.). The 3rd exception, isolated pulmonic stenosis, was the only case in which the relationship of high red cell (72 ml. per kgm.) and relatively standard plasma volume (44.1 ml. per kgm.) simulated that found in the tetralogy.

For the most part, the relative levels of plasma protein were within the standard range, as were the calculated values for total circulating plasma protein. Analysis of the data from the admittedly small number of cases fails to show any significant correlations between the total blood volume, red cell volume, total circulating hemoglobin or total circulating plasma protein and the hemoglobin percentage and hematocrit.

DISCUSSION

The findings in this study lend support to the point of view that the increased cell volume seen in the tetralogy of Fallot is due to a compensatory stimulation of the bone marrow consequent to the anoxia resulting from inadequate aeration of the circulating blood. Of the 3 prominent factors which might play a part in the development of the anoxemia, it is evident from our data that the septal defect is the least important. Thus, 2 of the cases of isolated septal defect (Nos. 221 and 223) showed significantly low hemoglobin and total cell volumes, one case (No. 222) showed approximately standard values, while the levels in the 4th case (No. 220) were only mildly elevated. The dextroposition of the aorta, on the other hand, must be considered as a possible factor, since it results in mixing of unaerated blood from the right side with aerated blood from the left ventricle. Blalock (19) has experimentally produced a similar mixing by a pulmonary arteriovenous anastomosis following lobectomy in the dog which led to anoxemia and polycythemia.

The most important factor in the production of the anoxemia and the compensatory polycythemia and hypervolemia, as seen in the tetralogy of Fallot, is probably the presence of pulmonic stenosis. This impedes the flow of venous blood into the pulmonic artery and forces the blood to pass into the systemic circulation. Talbott and his colleagues (20) have estimated that 75 per cent of the blood circulating through the heart may not go through the lungs. In addition, the increase in viscosity is a striking feature in the tetralogy of Fallot. We found, as did Talbott and his colleagues, that the blood was so viscous that it was withdrawn only with difficulty. This must impose a real burden upon the myocardium.

The combined action of these various factors results in a chronic anoxemia which resembles that seen in residence at high altitudes (21). The resulting polycythemia and hypervolemia are, in general, conditioned by the degree, duration, and continuity of the anoxic stimulus. As long as there is a marked discrepancy between the demands for oxygen by the tissues and the amount of oxygen furnished to them by the circulating blood, the stimulus continues to operate and the level of circulating red blood cells remains high. In spite of the increased hemoglobin level, the oxygen saturation in many patients is at a critical level of about 60 per cent. At this level, which corresponds to an oxygen saturation similar to that seen at an altitude of 4 miles, the margin of safety is small (19 and 20). The stimulus will be weakened when either the demand for oxygen is decreased or the supply increased. Prolonged bed rest thus

leads to a lessening of the stimulus and a decrease in the polycythemia. Likewise, the improvement in blood flow to the lungs and consequent increase in the oxygen supply to the tissues brought about by the Blalock procedure results in a decrease in the stimulus and in the polycythemia. The absence of significant polycythemia and hypervolemia in the Eisenmenger complex (Case 210) is to be expected on this basis, since in this patient much less venous blood passes into the aorta directly or by way of the septal defect; a greater portion enters the relatively wide orifice of the pulmonary artery to be oxygenated by the lungs; less cyanosis is observed, and in general the cardiodynamics more nearly approach the normal. In the same manner, no marked polycythemia or hypervolemia is found in patients with ventricular septal defects (Cases 220, 221, 222, and 223), since there is no flow of blood from the right to the left ventricle unless left ventricular failure is present. These patients, therefore, do not have arterial undersaturation and there is no stimulus to bone marrow from anoxemia. It is difficult to reconcile the finding of an increased red cell volume in isolated pulmonic stenosis (Case 209) with the fact that the arterial blood would not be expected to be undersaturated in this condition. However, the similarity of the plasma and cell volume changes in our cases of isolated pulmonic stenosis and Fallot's tetralogy suggests a possible similar etiological basis for the hypervolemia in these 2 diseases. We cannot explain the increase in plasma and cell volume in the case of patent ductus arteriosus.

The fact that the chronic anoxemia of the tetralogy of Fallot does not modify the erythropoietic activity permanently is of interest. Hurtado, Merino, and Delgado (21) have shown that normal individuals who have lived since birth at high altitudes and are brought down to sea level, show after some time, blood characteristics similar to those found in normal individuals who have always lived at sea level. Similarly, decrease of the anoxemia in patients with tetralogy by bed rest or the Blalock procedure results in a gradual change towards the blood picture found in the so-called "normal" individual. The fate of the red blood cells involved in this decrease awaits further study. The relatively gradual change and the absence of clinical icterus suggests that the superfluous red cells already in circulation are being destroyed while fewer red cells are being elaborated by the bone marrow.

The effectiveness of the polycythemia in the tetralogy seems to lie in the fact that it enables the patient to meet his resting needs for oxygen although it may not provide for much margin of safety. The large volume of viscous blood, however, imposes a rather severe strain on the heart which usually eventuates in congestive heart failure in the patient surviving to the 3rd or 4th decade, as cardiac reserve diminishes.

These studies are being continued. It would seem desirable to know much more about the underlying physiological adaptations of the circulation and respiration in congenital heart disease. Since among the most marked changes are those which occur in red cell volume, it would be of value to check further the validity of the dye-hematocrit method in these conditions by the carbon monoxide or radioactive-iron techniques. The material gathered to date, however, indicates the possible usefulness of blood volume determinations in supplementing the usual tests for the differentiation of the various types of congenital heart disease. The presence of an absolute erythrocytosis, as indicated by a significant increase in red cell volume without significant change in the plasma volume, should be interpreted as suggestive of the presence of pulmonic stenosis. The serial determination of the blood volume and total circulating hemoglobin should also serve as a valuable aid in indicating the optimal time for surgery and as a measure of the beneficial effects of surgical procedures.

CASE REPORTS

I. No. 208, W. M., 22 years

This patient gave a history of having been blue and having had clubbing of the fingers and curved nails since early childhood. Relatives stated he had been blue since birth. He had severe dyspnea and many dizzy and fainting spells occurring on slight exertion. He had an acute thrombophlebitis followed by chronic leg edema and ulceration in 1943. This responded well to treatment consisting of elimination of arterio-spasm by lumbar sympathetic blocks and compression dressings of elastic bandage or Unna's paste. In 1945 a high-low ligation for superficial varicosities was done on the right, after which the low incision healed very slowly. He was also admitted on one occasion with a diagnosis of "psychotic personality" and "gastric neurosis."

Physical examination on various admissions showed a fairly well developed adult white male. The skin was of a dusky blue color particularly marked in the ears and face. On exertion the cyanosis became more intense. No resting dyspnea was present, but exertional dyspnea was marked. Three-plus clubbing of the fingers and watch-crystalling of the nails was present. Examination of the chest revealed a loud precardial systolic murmur maximal over the pulmonic area. A systolic thrill over the same area was present. A 6-foot heart plate showed a pulmonary conus concavity and a small aortic knob. Electrocardiogram showed pronounced right axis deviation, and cardiography showed delay in right ventricle emptying. The aortic arch and descending aorta were demonstrated to be on the left when barium was introduced into the esophagus. Circulation time arm-to-tongue was 7 seconds with calcium gluconate. Determination of circulation time with ether in this patient resulted in a measurement of arm-to-face circulation time of 3.1 seconds as evidenced by severe burning of the face. The failure of this patient to smell the ether at any time after injection suggests that the amount of blood going to his lungs was very small as compared to his total blood volume. Arterial oxygen content was 18.35 per cent and venous 11.29 per cent. Venous oxygen capacity was 27.28 per cent. Venous pressure was 132 mms. of citrate; R.B.C.'s varied from 8 to 10 million. Other blood findings are given in Table I and Figure 2. Liver and kidney function tests were normal.

Treatment with semi bed rest from January to March, 1946, resulted in some improvement in exertional dyspnea and cyanosis, and resting cyanosis diminished. Attempted anastomosis of the right subclavian-pulmonary artery on May 9, 1946, by Dr. Alton Ochsner, was unsuccessful because of the relative shortness of the subclavian artery. Following 6 weeks of intermittent right carotid compression, anastomosis of the right common carotid-right pulmonary artery by the Blalock method was performed by Dr. Ochsner. Following this, the patient's cyanosis diminished for a few hours. He developed a left hemiplegia, however, and expired on the 3rd postoperative day.

II. No. 211, M. C., 6 years

The essential history on this boy, obtained from his mother, was that he had been blue since birth. The blueness was intensified by even slight exertion. He became short of breath upon slight exertion and as he became older had "falling out" spells on attempting exercise, such as running or climbing stairs. His mental development was normal and his physical development somewhat retarded. Positive physical findings were: (*1*) moderately underweight; (*2*) increased duskiness of face, ears, lips, finger-nails, markedly exaggerated on crying; (*3*) marked clubbing and watch-crystalling of fingers and toes; (*4*) loud precordial systolic murmur maximal over pulmonic area; (*5*) right axis deviation and sinus tachycardia on E.C.G.; and (*6*) R.B.C. of 6 to 8 million.

This patient was observed and treated with bed rest for a period of 2 months, during which time there was no change except questionably slight improvement of his exertional dyspnea. He, at no time, had findings suggestive of congestive failure. On July 23, 1946, a Blalock type anastomosis was done by Dr. Ochsner between the right subclavian and the right pulmonary artery. Immediate slight improvement in cyanosis resulted at this time. Little blood having been lost during the operation, it was thought advisable, in order to decrease the blood viscosity and because of danger of clotting, to withdraw 150 ml. of blood. Withdrawal of this amount caused the patient to go into severe secondary shock which was quickly overcome by re-administration of the blood.

The patient's convalescence was uneventful. Only a trace of finger and toe clubbing and watch-crystalling remains. At present he has no resting cyanosis and only slight exertional cyanosis. His exercise tolerance is much improved, his blood volume has dropped as previously noted (Figure 1). However, no murmur except the previously present pulmonic murmur can be heard.

The prompt secondary shock sustained by this patient when, following Blalock's suggestion (19), 150 ml. of blood was withdrawn postoperatively, raises the question as to the desirability of bleeding in these cases. There seems to be no reason to anticipate a sudden shrinkage of the vascular capacity so as to produce increased viscosity and clotting. In fact, as Blalock has reported and we have found, the changes towards "normal" occur gradually over a period of weeks. It is more likely that the trauma of the relatively long operation produces a widespread dilatation and temporary enlargement of the vascular bed, which certainly provides an adequate factor of safety during the early postoperative period.

SUMMARY

1. Plasma, red cell, and total blood volumes, total circulating hemoglobin, and plasma protein were studied in 7 cases of the tetralogy of Fallot. 4 cases of isolated interventricular septal defect, and 1 case each of probable Eisenmenger complex, isolated pulmonary stenosis, coarctation of the aorta, congenital mitral stenosis, and patent ductus arteriosus.

2. All patients with tetralogy of Fallot and the case of pulmonic stenosis showed a markedly increased blood volume due primarily to an increase in total red cell volume. Hematocrit and hemoglobin levels were also high. Plasma volume was at, or slightly above, standard levels.

3. Bed rest and production of a new shunt by the Blalock procedure in 2 cases resulted in a gradual decrease of red cell volume toward standard values.

4. Blood volume was at approximately standard levels in the cases of interventricular septal defect, Eisenmenger complex, and coarctation of the

aorta. The cases of congenital mitral stenosis and patent ductus arteriosus showed high blood volumes due to increases in plasma volume as well as red cell volume.

We are grateful to Miss Genie Burt and Dr. Blanche Jackson for their assistance and to Drs. V. Platou and Clarence T. Rae for their cooperation. The patients were hospitalized at Charity Hospital and Touro Infirmary.

BIBLIOGRAPHY

1. Blalock, A., and Taussig, H. B., Surgical treatment of malformations of heart in which there is pulmonary stenosis or pulmonary atresia. J. A. M. A., 1945, 128, 189.
2. Fallot, A., Contribution à l'anatomie Pathologique de al Maladie Bleue (cyanose cardiaque). Marseille Med., 1888, 25, 77, 138, 207, 270, 341, 403.
3. Abbott, Maude E. Atlas of Congenital Cardiac Disease. Am. Heart Assoc., New York, 1936, p. 60.
4. Glazebrook, A. J., Eisenmenger's complex. Brit. Heart J., 1943, 5, 147.
5. White, P. D., and Sprague, H. B., Tetralogy of Fallot; report of case in noted musician, who lived to his sixtieth year. J. A. M. A., 1929, 92, 787.
6. Meyer, P., Hamodynamik und Hamophysikochemie bein cinem Fall von Ventrikelseptumdefekt mit Pulmonalstenose; die Bedeutung der Hamoglobinvermehrung bei Sauerstoffmangel. Ztschr. f. klin. Med., 1932, 120, 341.
7. Blumenfeldt, E., and Wollheim, E., Zur klinischen Beurteilung angeborener Herzfehler. Klin. Wchnschr., 1927, 6, 396.
8. Weber, F. P., and Dorner, G., A case of congenital pulmonary stenosis, with special consideration of the nature of the secondary blood changes. Lancet, 1911, 1, 150.
9. Hallock, P., Polycythemia of morbus caeruleus (cyanotic type of congenital heart disease). Proc. Soc. Exper. Biol. & Med., 1940, 44, 11.
10. Gregersen, M. I., Practical method for determination of blood volume with dye-T-1824; survey of present basis of dye-method and its clinical applications. J. Lab. and Clin. Med., 1944, 29, 1266.
11a. Chapin, M. A., and Ross, J. F., Determination of true cell volume by dye dilution, by protein dilution, and with radioactive iron. Error of centrifuge hematocrit. Am. J. Physiol., 1942, 137, 447.
b. Lawson, H. C., Rappaport, D. B., and Ramirez, A., The recovery of injected dye and the yield of blood cells by perfusion of the cardiovascular system in barbitalized dogs. Am. J. Physiol., 1946, 147, 412.
c. Stead, E. A., Jr., and Ebert, R. V., Relationship of plasma volume and cell plasma ratio to total red cell volume. Am. J. Physiol., 1941, 132, 411.
d. Gibson, J. G., II, Weiss, S., Evans, R. D., Peacock, W. C., Irvine, J. W., Jr., Good, W. M., and Kip, A. F., The measurement of the circulating red cell volume by means of two radioactive isotopes of iron. J. Clin. Invest., 1946, 25, 616.
12. Root, W. S., Roughton, F. J. W., and Gregersen, M. I., Simultaneous determination of blood volume by CO and dye (T-1824) under various conditions. Am. J. Physiol., 1946, 146, 729.
13. Evelyn, K. A., Stabilized photoelectric colorimeter with light filters. J. Biol. Chem., 1936, 115, 63.
14. Barbour, H. G., and Hamilton, W. F., Falling drop method for determining specific gravity; clinical applications. J. A. M. A., 1927, 88, 91.
15. Weech, A. A., Reeves, E. B., and Goettsch, E., Relationship between specific gravity and protein content in plasma, serum, and transudate from dogs. J. Biol. Chem., 1936, 113, 167.
16a. Davis, L. J.: Determination of blood volume in man with Evans blue ("T-1824"). Edinburgh M. J., 1942, 49, 465.
b. Gibson, J. G., Jr., and Evans, W. A., Jr., Clinical studies of blood volume; relation of plasma and total blood volume to venous pressure, blood velocity rate, physical measurements, age, and sex in 90 normal humans. J. Clin. Invest., 1937, 16, 317.
c. Stewart, J. D., and Warner, F., Observations on severely wounded in forward field hospitals, with special reference to wound shock. Ann. Surg., 1945, 122, 129.
d. Taylor, H. L., Erikson, L., Henschel, A., and Keys, A., Effect of bed rest on blood volume of normal young men. Am. J. Physiol., 1945, 144, 227.
17. Brines, J. K., Gibson, J. G., Jr., and Kunkel, P., Blood volume in normal infants and children. J. Pediat., 1941, 18, 447.
18. Wintrobe, M. M., Clinical Hematology. Lea and Febiger, Philadelphia, 1942.
19. Blalock, A., Effects of an artificial ductus arteriosus on experimental cyanosis and anoxemia. Arch. Surg., 1946, 52, 247.
20. Talbott, John H., Coombs, F. S., Castleman, B., Chamberlain, F., Consolazio, W. V., and White, P. D., Record case of tetralogy of Fallot, with comments on metabolic and pathologic studies. Am. Heart J., 1941, 22, 754.
21. Hurtado, A., Merino, C., and Delgado, E., Influence of anoxemia on hemopoietic activity. Arch. Int. Med., 1945, 75, 284.

THE RELATION BETWEEN THE SERUM UREA CONCENTRATION AND THE PROTEIN CONSUMPTION OF NORMAL INDIVIDUALS [1]

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When a patient with renal disease is found to have a concentration of urea in his blood that is higher than usual we would like to be able to derive from the degree of increase in concentration some idea as to the degree of decrease in the urea-excreting function of his kidneys. In the individual patient this cannot safely be done. True, when we plot the blood urea concentrations of a large number of patients against their urea clearances, a general relation does emerge (1, 2). However, in any one of such a group of patients the prediction from concentration to clearance may fall dangerously far from the truth. This is inevitable because the concentration of urea in the body is determined not only by how much urea runs out of the system through the kidneys but also by how much urea runs into it from the liver. When more runs out than runs in, the concentration falls to a lower level which is maintained as long as the discrepancy persists. When more runs in than runs out the concentration level rises and remains high as long as the excess of in-flow over out-flow continues. Unlike salt, the concentration of urea is confined within no narrow zone of variation by any regulatory mechanism. Urea is a substance to which the body is chemically and physically indifferent. It is an end product of protein metabolism and participates in no chemical reactions. Except in the kidney it has no osmotic effect because it is distributed evenly through the water of all organs and tissues. So we need not be surprised when we find, as we do, that there is a wide scatter in the urea concentrations in the blood of normal individuals (3, 4). In spite of wholly normal renal function we shall continue to find this high variability until we make measurements on normal subjects who are taking the same amounts of protein in their food; and in our patients, whose renal function is not normal, we shall not be able to derive reliable judgments as to the renal significance of our blood urea determinations until we know their food protein consumption and can exclude any pronounced deviation from the usual rate of endogenous protein catabolism.

When an acutely ill patient first reaches a hospital it is generally recognized that even a quite high blood urea concentration cannot be taken as conclusive evidence of renal failure. There is always the chance that this may be an example of "extra-renal azotemia," a term used to cover any sudden and pronounced increase in urea formation by the liver, whether the amino acids from which it is derived come from the gastrointestinal tract, as in massive blood loss into the gut, or from a rapid breakdown of the protein of the body itself, as in certain patients with obstruction of the small intestine. The conditions that lead to such explosive disintegrations of large amounts of protein are not often found in ambulatory patients. In them, we may suppose, differences in the rate of urea formation arise mainly from differences in the quantities of protein they take as food. It is for this group, and particularly for out-patients with renal disease, that we might get more information from blood urea measurements if only we were able to discount this protein consumption variable. That cannot be done until we measure the effect of variation in protein consumption on the blood urea concentration of normal individuals.[2]

[1] This work was made possible by a grant from the Nutrition Foundation, Inc.

[2] In accordance with clinical usage we speak of blood urea concentrations although the determinations were actually carried out on plasma or serum. When whole blood is used the urea concentration is found to be about 1 per cent less than in serum because the red cells contain less water than the serum. This is true only when the arginase in the red cells has been destroyed by heat, before the urease is added. Otherwise determinations on whole blood give a variable plus error which we believe arises because the arginase in the red cells produces urea from arginine in the urease (5).

The data given in this paper were collected with that end in view.

Method

1. *The conditions under which the observations were made*

The experiment was made possible through the intelligent and devoted cooperation of 2 groups of people: first, the 10 medical students who were the subjects, and, second, the student dietitians who designed and prepared weighed diets containing varying quantities of protein. The effect of 3 levels of protein consumption was studied. The first contained an amount of protein not much more than is required for nitrogen equilibrium (6). This was obtained by giving each subject 0.5 gram of protein for every kilogram of his body weight (with clothes). The second represented a medium protein consumption and provided 1.5 grams of protein per kgm. The third, a high protein diet, supplied 2.5 grams of protein for every kgm. of body weight. The subject who weighed 80 kgm. thus received, first, 40 grams, then 120 grams, and, finally 200 grams of protein a day.[3] The calories in all cases exceeded 2,000 a day. There was no restriction of water. The subjects continued with their daily work. Blood was collected on the 5th and 6th days of the consumption of each level of protein. On the 5th day the first blood was taken at 7:15 a.m. before breakfast at 7:30 a.m., then at 11:45 a.m. before lunch at 12 noon, at 4:45 p.m. before dinner at 5 p.m., and at 9:45 p.m. before going to bed at 10 p.m. On the 6th day observations were made at 7:15 a.m. and at 11:45 a.m. just before breakfast and lunch. Between each diet there was a period during which whatever food the subjects wanted was taken. The 0.5-gram level was taken first, then the 1.5, and then the 2.5-gram level. After a 2-month interval the 0.5- and the 2.5-gram diets were repeated. We thus obtained 120 serum urea determinations on 0.5 gram, 60 on 1.5 grams and 108 on 2.5 grams, a total of 288 observations.

2. *The method of measurement*

A urease-aeration-titration method was used (7). In spite of constant care and reiterated checking with known urea solutions we suspect that our data may be marred by the occasional intrusion of unsuspected technical errors. We have reason also to think that there is a constant minus eror of nearly 3 per cent arising from unavoidable loss in the transfer of ammonia. In spite of these imperfections we believe the method adequate for our purpose. That this is so is indicated, though not proved, by the general agreement between our present results and the results we obtained with the same method in 1940. In these earlier measurements the subjects were different (10 residents and internes) but the protein variation was the same. Unfortunately, in 1940, blood was taken only once a day, so that the comparison has to be restricted to the measurements made at 11:45 a.m. on the 5th day of each diet in 1941 and the measurements made at the same time of the 5th day in the present series. The degree of agreement is shown in Table I.

We cannot say how much of the divergence between the 2 sets of observations shown in Table I should be ascribed to inconsistency in the technique of measurement. It is enough to observe that the sum of the technical and of all other reasons for difference do not prevent us from

TABLE I

Comparison of blood urea concentrations measured at 11:45 a.m. in 1940 with concentrations measured at 11:45 a.m. in the present series using the same method

1941 measurements				Present measurements			
Subject	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.	Subject	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.
	mgm. per 100 ml.	*mgm. per 100 ml.*	*mgm. per 100 ml.*		*mgm. per 100 ml.*	*mgm. per 100 ml.*	*mgm. per 100 ml.*
1	19.9	38.0	43.0	A	18.6	42.3	42.5
2	19.9	26.5	39.7	B	15.6	35.3	43.4
3	23.2	26.5	39.7	C	20.0	40.6	45.2
4	19.9	33.1	34.6	D	26.2	54.7	55.8
5	23.2	38.0	39.7	E	18.6	40.6	46.9
6	21.5	33.1	44.7	F	20.4	42.3	44.3
7	23.2	44.7	57.9	G	19.1	40.6	43.4
8	23.2	36.4	48.0	H	18.2	38.8	43.4
9	23.2	46.3	53.0	I	22.1	44.1	41.6
10	23.2	34.7	46.3	J	18.6	38.8	40.7
Average	22.0	35.7	44.6		19.7	41.8	44.7

TABLE II

Individual averages from observations made at all times of day on varying levels of protein concentration

Subject	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.	Individual averages for the 3 diets
	mgm. per 100 ml.	*mgm. per 100 ml.*	*mgm. per 100 ml.*	*mgm. per 100 ml.*
J	18.3	37.8	40.9	32.3
B	17.6	39.3	40.4	32.4
H	17.8	34.9	45.2	32.6
A	17.9	36.9	44.4	33.1
F	19.1	36.9	43.5	33.2
G	19.8	36.6	44.2	33.6
C	18.9	35.8	47.6	34.1
I	20.3	40.5	43.0	34.6
E	18.7	39.6	49.2	35.8
D	24.4	48.1	56.6	43.1

[3] The body weights of our subjects varied from 65 to 83 kilograms. Unquestionably somewhat more precise results would have been obtained if we had apportioned the protein in terms of some power of the body weight. The method we used is based on the not quite correct supposition that there is a relation of direct proportionality between body weight and protein requirement. We used it because this is the simple first approximation that we

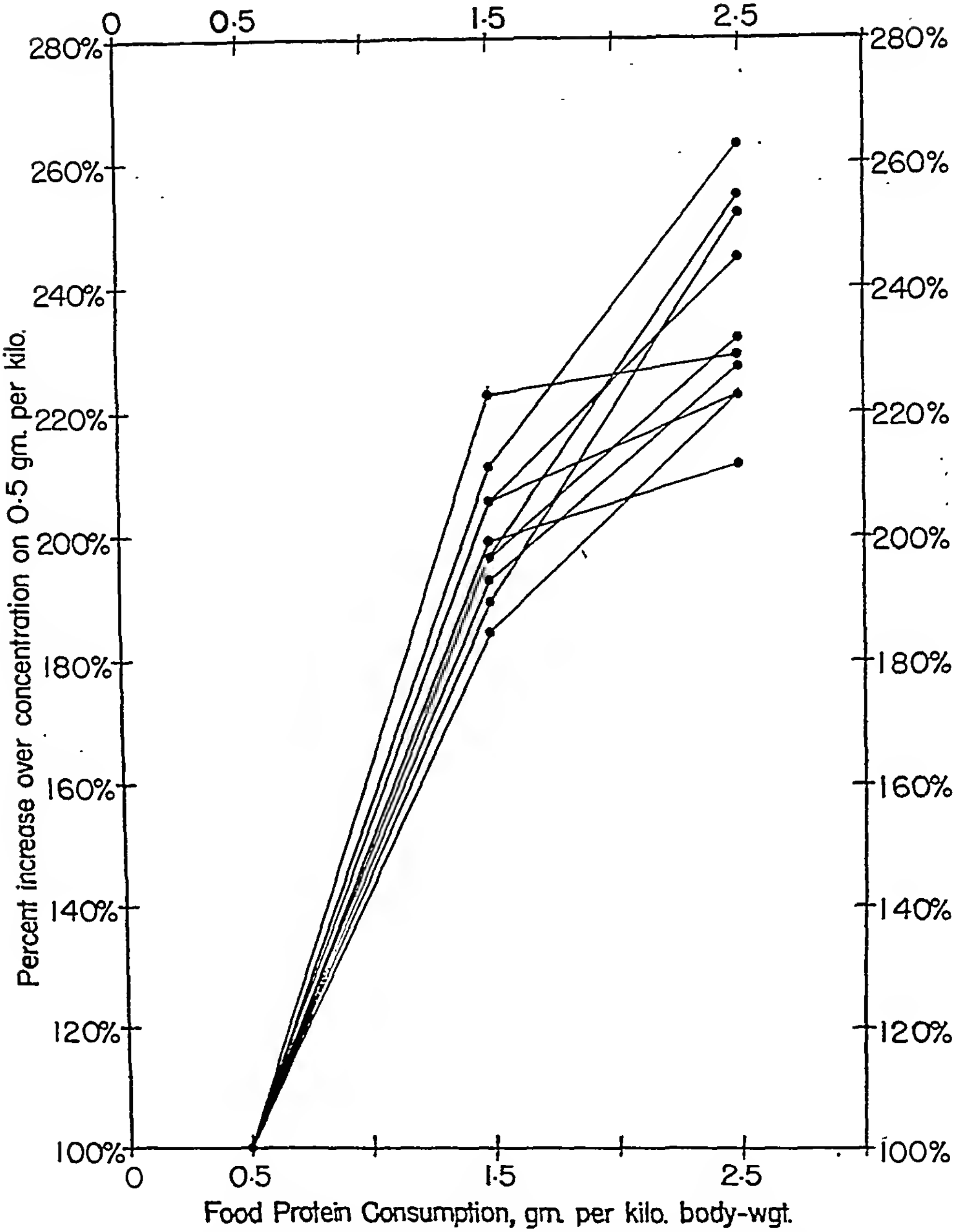

Fig. 1. The Relation between the Serum Urea Concentration and the Protein Consumption of Normal Individuals

concluding that in both of these groups of normal subjects the blood urea concentration rises as the protein consumption increases and is more than twice as high on the 2.5 as on the 0.5 gram of protein per kilogram diet.

3. *The individual variability of the subjects*

There are indications of individual peculiarities in the 20 subjects dealt with in Table I, but no conclusion can be drawn from these fluctuations because the number of observations on each subject is too small. We can get a better idea as to how much one individual may differ from another in his reaction to increase in protein consumption from the present series of observations. For each subject we have 12 measurements on 0.5 gram, 6 on 1.5 grams, and 11 or 10 on 2.5 grams of protein per kilogram. The averages for each individual are given in Table II and the percentage increases above the concentration on the 0.5-gram level are shown in graph form for each of the 10 subjects in Figure 1.

Though every subject has an increase in concentration with increase in protein, these results reveal considerable individual differences. It does not seem to us, however, that the question as to the probability that these differences arise from factors constant for each subject is one that our data can answer with sufficient dependability to give a result of practical value. For the moment it is enough to note that, if we take the concentration observed in each subject on the 0.5-gram protein diet as 100 per cent, these normal individuals responded to the 1.5-gram protein diet by increases in concentration that varied from 185 per cent to 223 per cent and from 212 per cent to 263 per cent when 2.5 grams of protein per kgm. was taken.

For clinical purposes it is most important to know how great is the individual variation in concentration in normal subjects taking the same amount of protein. This question is answered in the last column of Table II where we give the averages of 29 observations on 8 subjects, and 28 on the other 2, at all times of day and under all diets. The subjects have been arranged in the order of magnitude of their averages.

In 9 subjects there is a close concordance in blood urea concentration, with a range of from 32.3 to 35.8 mgm. per 100 ml. The subject D, however, has an average of 43.1 mgm. per 100 ml. Yet he was what we call "normal," that is, he considered his health good, he was working hard, and, in particular, was free from any sign of renal disease. There was nothing unusual in his reaction to increase in food protein, for it happens he falls close to the center of the stream of variation shown in Figure 1. It is fortunate that we happened to include this subject, for if some other student, who was like the rest of the group, had taken his place, the reported variability in blood urea concentration would have been narrowed. It is obvious that measurements of normality based on 10 subjects have only a preliminary and tentative significance.

RESULTS

We neglect all variables except the change in protein consumption and present only the averages and their standard deviations on 0.5, 1.5 and 2.5 grams of protein per kgm. body weight. This means that we include the variability arising from the fact that the blood samples were taken at various times during the two 24-hour cycles within the variation induced by the protein. We prefer to merge these 2 distinguishable effects because we intend to use our measurements for the interpretation of the urea concentration of patients no matter at what time of day we take their blood.

In Table III we give the averages and their standard deviations. In Figure 2, the averages are plotted against the protein consumption and the area lying between the averages plus or minus twice the standard deviations is indicated. This area is delimited by lines because we want to use it for the interpretation, from blood urea concentration measurements, of the renal status of patients who are taking any quantity of protein between 0.5 and 2.5 grams per kgm. The lines are broken in order to indicate that when the protein consumption is other than 1 of the 3 quantities at which our measurements were made we are dealing with assumed and not with measured variabilities.

TABLE III

Averages and variabilities of blood urea concentrations on varying and on all levels of protein consumption

	Food protein consumption			All observations on all 3 diets
	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.	
	mgm. per 100 ml.	*mgm. per 100 ml.*	*mgm. per 100 ml.*	*mgm. per 100 ml.*
Average blood urea concentration	19.3	38.6	45.5	33.1
Number of observations	120	60	108	288
Standard deviation	2.9	7.1	7.2	13.3
Standard error	0.27	0.91	0.69	0.78
	per cent			
Coefficient of variation	15.2	18.3	15.7	40.1

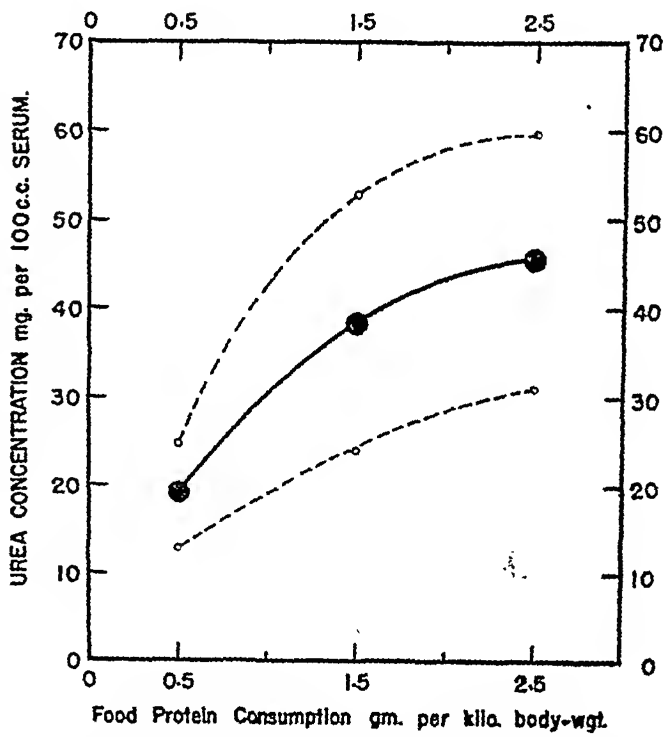

FIG. 2. THE RELATION BETWEEN THE SERUM UREA CONCENTRATION AND THE PROTEIN CONSUMPTION OF NORMAL IDIVIDUALS

DISCUSSION

The average of all the 288 measurements is 33 mgm. Of these 95 per cent lie between 7 mgm. and 60 mgm. of urea per 100 ml. of blood. This result is in conformity with what has already been observed (4, 5). All it does is to demonstrate, more precisely than before, how wide is the variability in the concentration of urea in the blood of apparently healthy young adults who present no evidence of any defect in renal function. If this were all, we should have to conclude that, even for ambulatory patients with renal disease, a measurement of blood urea concentration was of little value, apart from its use in revealing the more extreme degrees of renal failure.

Now, however, the effect of variation in protein consumption has been measured in normal individuals. As we go from 0.5 to 1.5 to 2.5 grams of protein per kgm. their blood urea concentration rises from 19 to 39 to 45 mgm. per 100 ml. At each of these levels of protein consumption the variability is much lower than the 40 per cent coefficient of variation that characterizes the whole series. It is reduced to 15 per cent at 0.5 gram, 18 per cent at 1.5 grams, and 16 per cent at 2.5 grams. This means that we now have measurements on which we can base our clinical judgments with greater assurance.

The prerequisite for the use of these standards is a knowledge of the quantity of protein the patient takes. But this is what is already known by everyone who believes that the protein consumption of a patient with renal disease is an important factor in his treatment. Many of our patients are living on self-measured diets that contain 0.5 gram of protein per kgm. We have reason to know that these measurements have a reasonable precision. That is a matter that can be verified by periodic determinations of their rate of urea excretion. If, in such a patient, we find a blood urea concentration of 40 mgm. per 100 ml. we can be almost certain that he has a urea clearance well below normal. We know that because in Figure 1 we see that on 0.5 gram of protein per kgm. only about 2 per cent of people with unimpaired renal function have urea concentrations that exceed 25 mgm. per 100 ml. If, as the months and years go by, while the same diet is maintained, we find this patient's concentration rising from 40 to 50 to 60 mgm., or falling from 40 to 30 to 20 mgm., we now have some warrant for the supposition that his urea clearance has changed in an approximately proportionate inverse manner.

Whatever average quantity of protein a patient loses in his urine is added to his diet. In some this may amount to 10, in others to 15 and in still others to 20 grams of protein a day. Thus the protein taken by an 80-kgm. patient may be increased from 40, to 50, to 55 or to 60 grams of protein a day, that is from 0.5 to 0.63, to 0.69, to 0.75 gram of protein per kgm. In such patients there are reasons, and some of them are more than statistical, why we cannot have the same assurance in the interpretation of urea concentrations measured at levels of protein consumption other than those at which our determinations were made. Until we have more extensive information, however, our best guess is to assume smooth curves between the measured points in Figure 1. Then, if a patient is taking 0.75 gram of protein per kgm., we shall be guided in the estimation of the urea-excreting function of his kidney by the supposition that, with unimpaired function, the average concentration would be 25 mgm. and that in only 2 per cent of all such instances would the concentration be more than 33 mgm. per 100 ml.

In theory, we can now predict the urea clearance of any ambulatory patient whose protein consumption is known. In practice, however, we have to remember that 10 medical students are not an adequate sample of all people whose renal function is supposedly intact. More than that, the theory involves the assumption that the rate and variability of "endogenous" protein catabolism in patients with renal disease is similar to that which existed in our normal sample. That is a supposition that has never been tested. Nevertheless we find these normal standards useful, even though we are aware that our "predictions" are not much more than clinical judgments, influenced in an indeterminate manner by considerations arising from a concrete knowledge of the particular circumstances that surround each one of our patients.

CONCLUSIONS

1. The average of 288 determinations of the urea concentration in the blood of 10 medical students was 33 mgm. per 100 ml. The range of variation

that included 95 per cent of the measurements went from 7 to 60 mgm. of urea per 100 ml.

2. When the subjects took 0.5 gram of food protein per kgm. body weight the average of 120 blood urea concentration determinations was 19 mgm. per 100 ml., and 95 per cent lay between 13 and 25 mgm. per 100 ml. On a diet containing 1.5 grams of protein per kgm. the average of 60 determinations was 39 mgm., and 95 per cent fell between 25 and 53 mgm. per 100 ml. When 2.5 grams of protein per kgm. was taken, the average of 108 determinations was 45 mgm. and the same proportion was included in a range from 31 to 60 mgm. per 100 ml.

BIBLIOGRAPHY

1. MacKay, E. M., and MacKay, L. L., The relation between the blood urea concentration and the amount of functioning renal tissue. J. Clin. Invest., 1927, 4, 127.
2. Van Slyke, D. D., McIntosh, J. F., Möller, E., Hannon, R. R., and Johnson, C., Studies of urea excretion. VI. Comparison of the blood urea clearance with certain other measures of renal function. J. Clin. Invest., 1930, 8, 357.
3. Addis, T., and Watanabe, C. K., The causes of variation in the concentration of urea in the blood of healthy adults. Arch, Int. Med., 1917, 19, 507.
4. MacKay, E. M., and MacKay, L. L., The concentration of urea in the blood of normal individuals. J. Clin. Invest., 1927, 4, 295.
5. Addis, T., An error in the urease method for the determination of urea. Proc. Soc. Exper. Biol. & Med., 1928, 25, 365.
6. Hegsted, D. M., Tsongas, A. G., Abbott, D. B., and Stare, F. G., Protein requirements of adults. J. Lab. & Clin. Med., 1946, 31, 261.
7. Addis, T., Urea determinations in blood and urine. J. Lab. & Clin. Med., 1925, 10, 402.

THE SERUM CREATININE CONCENTRATION OF NORMAL INDIVIDUALS [1]

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In the preceding paper (1) it is shown that the blood urea concentration of subjects with intact renal function increases as their protein consumption increases. The conclusion is drawn that a relatively dependable inference from urea concentration to renal function requires a knowledge of the amount of protein a patient is taking and, in addition, a clinical situation that warrants us in excluding any sudden and pronounced increase in protein catabolism. In general, this second requirement is met when we deal with ambulatory patients, but at the first visit of every new patient the first requirement is lacking. Under the circumstances it would be well if we could measure some substance, other than urea, the concentration of which is uniquely determined by the functional capacity of the kidney. Unlike urea, this substance should have a rate of formation that is constant and that is unaffected by variation in food consumption. Creatinine may be such a substance. Its rate of flow into the blood stream is said to depend on the rate of transformation of creatine into creatinine (2). Its rate of excretion is independent of the amount of food protein that is eaten (3) and remains the same whether the muscles are active or at rest (4). We have therefore measured the variability of serum creatinine concentrations in individuals without proteinuria whose renal function was presumably unimpaired.

METHOD

1. *The conditions under which the observations were made*

The same subjects, the same diets, the same blood collections were used for the serum creatinine as for the blood urea concentration measurements (1). But within this identity of conditions there is concealed a difference that exists for creatinine but not for urea. At all levels of protein intake the food was devoid of preformed urea, but it was not free from preformed creatinine. When 0.5 gram of protein per kgm. was taken there was no meat in the diet and so the food was creatinine-free. But on the 1.5-gram and still more on the 2.5-gram level there was an increasing consumption of meat, fish, and chicken. In cooking, some of the creatine of muscle is converted into creatinine, in an amount that varies, presumably, with time, temperature, and pH (5). Since the unaltered creatine of the meat does not appear in the urine as creatinine (6) and since an increase in protein consumption does not increase creatinine excretion (3) we may derive from the 24-hour rates of creatinine excretion of our subjects an indication as to the quantity of creatinine formed from creatine in cooking. These rates are given in Table I.

TABLE I

Rate of creatinine excretion on a creatinine-free diet (0.5 gram protein per kgm.) and on diets containing increasing amounts of cooked muscle tissue (1.5 and 2.5 grams protein per kgm. body weight)

Averages from groups of 10 subjects. Rates corrected for a body weight of 70 kgm.

	0.5 gram per kgm. creatinine	1.5 grams per kgm. creatinine	2.5 grams per kgm. creatinine
	mgm. per 24 hours		
1940 observations	1638	1766	1903
Present observations	1597	1743	1835

If we take the average of both rates of creatinine excretion on the 0.5-gram diet as representing the endogenous creatinine, we note in Table I that this is increased by 137 mgm. on the 1.5-gram and by 252 mgm. on the 2.5-gram protein diet. This increase we may reasonably attribute to the addition of exogenous creatinine formed in cooking. The point is worth mentioning because it may seem unfortunate that the conditions we chose for our observations include a variable that may prevent us from obtaining a true measure of the variability of serum creatinine concentrations in normal individuals. This would be true enough if the variability determination were our end, instead of being a means to our end of establishing a standard of normality for new patients, many of whom will certainly have taken varying amounts of cooked meats before we see them.

With the exception of the 9:45 p.m. collection, blood was drawn just before breakfast, lunch, and dinner, that is to say at as long a period as possible since the preceding meal. Preformed creatinine is rapidly excreted

[1] This work was made possible by a grant from the Nutrition Foundation, Inc.

and so the maximum increase in serum creatinine concentration may have occurred before we got our blood samples. We measured the serum creatinine concentration in 32 nurses about an hour to an hour and a half after a lunch that contained soup and meat. The average concentration was 0.98 mgm. per 100 ml. The average obtained in 16 nurses who had taken no soup or meat was 0.91 mgm. per 100 ml. From the standard errors of these means it was calculated that such a difference would arise from chance in about 5 of every 100 repetitions of the experiment. It is thus probable that a somewhat greater variability than the one we report in Table IV would have been found if we had made our observations at times other than those we selected.

We shall restrict ourselves to the present series of observations in determining the variability of serum creatinine concentrations. The group involved was distinguished from the 1940 group by the fact that one of the subjects was a woman. Though the point has never been established beyond reasonable doubt there is some indication that creatinine concentrations are a little lower in women than in men (7). In our series we have 257 observations on our 9 male subjects and 29 on E, our single woman subject. The mean for the men was 1.03 mgm. per 100 ml. and for subject E 0.95 mgm., but, from the standard errors of these means, it can be shown that the difference between them might readily arise by chance. We conclude that, for our particular purpose, we may include subject E and use the total variability in judging the significance of observed concentrations in both male and female patients.

2. *The method of measurement*

The photoelectric cell has facilitated the measurement of the color of creatinine picrate but even so the order of precision is not high for normal serum creatinine concentrations. When tungstate filtrates are used, the color is so faint that the limit of capacity of the photoelectric cell in the Evelyn colorimeter is approached. In 1940 we used the standard Folin Wu method (8) except that we used an Evelyn instead of a Duboscq colorimeter. In the present series of observations 2 modifications were introduced. We took note of Peter's (9) statement that a 1.175 per cent solution of purified picric acid was preferable to the saturated solution usually used. At the suggestion of Phillips (10) all the reagents were brought to the same temperature before the reaction was started in a water bath that varied between 24.5° and 26.2° C., and this temperature was maintained until the color was read exactly 10 minutes after the reaction was initiated. When we compare those of the 1940 measurements with those of the present measurements which are strictly comparable it becomes clear that there is a difference between them which we can attribute only to these changes in technique.

The difference becomes apparent when we use the information given in Table IV. All of the examples of our present measurements fall within the range given by the average, 1.02 mgm., plus and minus twice the standard deviation of 0.118 mgm.; but 5 of the 29 measurements

TABLE II

Comparison of serum creatinine concentrations measured in 1940 with concentrations measured in the present series in serum taken at the same time on the 5th day of each diet

1940 measurements				Present measurements			
Subject	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.	Subject	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.
	mgm. per 100 ml.				mgm. per 100 ml.		
1	0.99	1.05	0.99	A	1.05	0.90	1.07
2	1.23	1.28	1.19	B	1.19	1.22	1.09
3	1.12	1.15	1.13	C	0.99	1.15	1.14
4	1.05	1.06	0.97	D	1.06	1.13	1.15
5	1.20	1.20	1.07	E	0.89	1.02	0.95
6	1.10	1.15	1.07	F	1.15	1.09	1.05
7	1.07	1.15	1.05	G	0.92	1.05	1.02
8	1.36	1.32	1.21	H	0.96	1.00	0.99
9	1.30	1.32	1.24	I	1.01	1.07	1.13
10	—	1.12	0.99	J	0.95	0.93	1.01

made in 1940 exceed 1.26 mgm., which is the upper limit of this range. For our purpose this is important because it is apparent that the standard we have set up is applicable only to measurements made with the particular technique we have described.

We have made these color determinations because we believe they measure creatinine. But if all we have been doing is to determine the variability of a color produced by various unknown chromogens there is no reason that we should suppose the resulting color to have any necessary reference to the kidney nor need there be any propriety in our proposal that we should take an increase in this color as a ground for questioning renal creatinine-excreting capacity. What direct evidence we have on this point, though of necessity it is neither statistically convincing nor wholly conclusive, is due to the development by Miller and Dubos (11) of 2 bacterial enzyme systems that specifically destroy creatinine. Having found an average serum or plasma "creatinine" concentration of 1.09 mgm. per 100 ml. in 15 normal individuals, they observed that these enzymes did away with a color equivalent to that produced by 0.99 mgm. of creatinine per 100 ml. There is so little difference between 1.09 and 0.99 mgm. that we are left free to suppose that all of the color might have been due to creatinine[2] But, however that may be, our principal reason for confidence in a relation between the color and renal function is the indirect evidence provided by the, for the most part, unrecorded experience of a multitude of clinicians who have watched

[2] It should be noted that in the serum of uremic patients Miller and Dubos found that not all of the color could be accounted for by creatinine (12). If this is confirmed we shall have to remember that at the higher levels of creatinine retention we cannot expect any direct relation between the degree of renal failure and the degree of the color we measure.

the "creatinine" concentrations of their patients' sera gradually rise as the functional capacity of their kidneys slowly fell.

3. *The individual variability of the subjects*

In Table III we give the averages for each subject, derived usually from 12 determinations on the 0.5-gram, 6 on the 1.5-gram and 12 on the 2.5-gram level of protein consumption. The averages for each subject for all 3 diets is given in the last column of this table, arranged in order of ascending magnitude. There is a gradual increase from 0.93 mgm. to 1.10 mgm. per 100 ml. and no one subject stands out as singular. Subject D, whose blood urea concentration was so high relative to the others (1), falls here well within the range of individual variation.

TABLE III

Individual average serum creatinine concentrations from observations made at all times of day on varying levels of protein consumption

Subject	Food protein consumption			
	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.	Individual averages for the 3 diets
	mgm. per 100 ml.			
G	0.92	0.92	0.95	0.93
H	0.96	0.88	0.94	0.93
E	0.91	0.96	0.99	0.95
J	1.05	0.98	0.98	0.98
A	1.06	1.03	1.01	1.03
B	1.08	1.01	1.05	1.05
C	1.07	1.11	1.09	1.09
D	1.07	1.14	1.08	1.09
F	1.13	1.04	1.08	1.09
I	1.05	1.13	1.13	1.10

RESULTS

The measurements given in Table IV show quite decisively that variation in protein consumption has no effect in the serum creatinine concentration. Our standard can therefore rest on all the 286 determinations. The average is 1.02 mgm. per 100 ml. and the standard deviation is 0.118 mgm. If we add twice the standard deviation to the average we have an upper range from 1.02 to 1.26 mgm. within which all but about 2 per cent of determinations will lie unless some other factor intervenes that did not exist in the subjects or in the conditions that we observed. The nature of the factor remains, of course, a question for clinical judgment.

TABLE IV

Averages and variabilities of serum creatinine concentrations on varying and on all levels of protein consumption

	Food protein consumption			
	0.5 gram protein per kgm.	1.5 grams protein per kgm.	2.5 grams protein per kgm.	All observations on all 3 diets
	mgm. per 100 ml.			
Average serum creatinine concentration	1.020	1.020	1.030	1.020
Number of observations	110	60	116	286
Standard deviation	0.126	0.123	0.106	0.118
Standard error	0.012	0.016	0.010	0.007
	per cent			
Coefficient of variation	12.4	12.1	10.3	11.5

DISCUSSION

Figure 1 contrasts the behavior of creatinine and urea as the protein consumption of normal individuals is increased.

With creatinine-concentration measurements we are delivered from the effect of a variable that obscures the significance of the urea or non-protein nitrogen determinations. We thus have a measure that we can use with assurance in the numerous clinical situations in which we have no precise information as to the rate of urea formation.

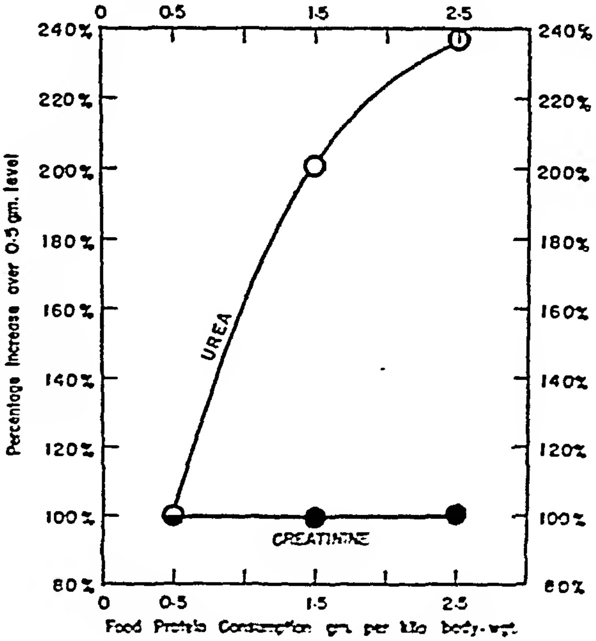

FIG. 1. FOR SERUM CREATININE CONCENTRATION OF NORMAL INDIVIDUALS

CONCLUSIONS

1. The average of 286 determinations of creatinine concentration in the serum of 10 medical students was 1.02 mgm. per 100 ml. The range of variation that included 95 per cent of the measurements extended from 0.78 mgm. to 1.26 mgm. per 100 ml.

2. Variation in protein consumption from 0.5 to 2.5 grams protein per kgm. body weight had no effect on the serum creatinine concentration.

We are indebted to Dr. Horace Gray for advice and help in the statistical treatment of the data.

BIBLIOGRAPHY

1. Addis, T., Barrett, E., Poo, L. J., and Yuen, D. W., Relation between serum urea concentration and protein consumption of normal individuals. J. Clin. Invest., 1947, 26, 869.
2. Hunter, A., Creatine and Creatinine, Monographs on Biochemistry. Longmans, Green and Co., London, 1928, p. 147.
3. Folin, O., Laws governing the chemical composition of the urine. Am. J. Physiol., 1905, 13, 66.
4. van Hoogenhuyze, C. J. C., and Verploegh, H., Beobachtungen über die Kreatininausscheidung beim Menschen. Zeit. f. physiol. Chem., 1905, 46, 415.
5. Zickelbein, U., Uber die Kreatin-Kreatininausscheidung mit dem Harn bei fleischfreier und fleischhaltiger Nahrung. Zeit. f. d. ges. Exp. Med., 1933, 87, 112.
6. Afklercker, K. O., Zur Frage der Kreatin—und Kreatininausscheidung beim Menschen. Beitr. chem. Physiol. und Path., 1906, 8, 59.
7. Hunter, A., and Campbell, W. R., The amount and the distribution of creatinine and creatine in normal human blood. J. Biol. Chem., 1918, 33, 169.
8. Folin, O., and Wu, H., A system of blood analysis. J. Biol. Chem., 1919, 38, 81.
9. Peters, J. H., The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., 1942, 146, 179.
10. Phillips, R. A., Personal communication.
11. Miller, B. F., and Dubos, R., Studies on the presence of creatinine in human blood. J. Biol. Chem., 1937, 121, 447.
12. Miller, B. F., and Dubos, R., Determination by a specific enzymatic method of the creatinine content of the blood and urine from normal and nephritic individuals. J. Biol. Chem., 1937, 121, 457.

A CLINICAL METHOD FOR THE APPROXIMATE DETERMINATION OF SERUM CREATININE CONCENTRATION

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In our two preceding papers (1, 2) we have given measures of the variability of blood urea and serum creatinine concentrations in normal individuals who were taking known amounts of food protein. These standards have been found useful in forming an opinion as to the renal status of ambulatory patients with proteinuria. As time goes by and successive measurements are made we are able to judge the direction of change in the functional capacity of their kidneys. Such data are of primary clinical value because they are elements in the more complex judgments on which therapeutic decisions depend. But our own experience has forced us to conclude that these methods require supplementation by some procedure more in consonance with the immediate and practical requirements of day to day clinical work. Every day, in every clinic for renal disease, there are apt to be several new patients in whom an answer to the question as to the extent of their renal lesion is needed. This is not usually obtainable from their histories and physical examinations or from the state of their urines. At best these methods may suggest the existence of suburemic or uremic states, but at times they fail us altogether. It is the determination of the concentration of creatinine in the serum that transforms these indications into knowledge on which treatment can be based. Now, in theory, there is no reason why a precise measurement of creatinine cannot be obtained within half an hour or so of drawing the patient's blood. But this determination involves a complete separation from the patient and an absorption in technique that may be inadvisable or impossible. It is then that we need a quick approximate measure of the serum creatinine level to be used for the purpose of general orientation and immediate preliminary treatment.

We happened one day to observe that when the alkaline sodium picrate used for the determination of creatinine was added to whole serum the mixture remained quite clear. As the tube was watched, the orange color of creatinine picrate was seen to appear but as time went on the color deepened so that a few hours later it had become a deep orange, far deeper than the color derivable from the creatinine in the serum we were using. It seemed that there must be some chromogen other than creatinine in whole serum. The two colors can, however, be distinguished. The creatinine color develops quickly, even faster in serum than in a protein-free filtrate from the same serum, whereas the non-creatinine color develops very slowly. In Figure 1 it is shown that within 6 minutes after the addition of alkaline picrate to serum the rate of color formation has already become asymptotic. By making the determinations at 6 minutes we were thus able to measure the quantities of creatinine we added to serum.

In the clinical method we describe, the creatinine color in the serum is read against potassium dichromate standards. The comparison is facilitated by diluting the standard alkaline picrate solution with an equal volume of water. This solution is conveniently prepared by adding 1 ml. of 10 per cent sodium hydrate to 5 ml. of 1.175 per cent picric acid, and diluting with water to 12 ml. If care is taken to avoid parallax errors this can be done in a 15-ml. graduated centrifuge tube, adding the required volumes to the mark by means of capillary pipettes. One ml. of the thoroughly mixed alkaline picrate is then added to 0.5 ml. of serum and an interval timer is set to ring at 6 minutes. The mixture of serum and picrate is then poured into a 4- by ½-inch test tube similar to the sealed tubes in which the potassium dichromate standards are kept. Tubes that have the same or almost the same diameter, by caliper measurement, and that are made of the same colored glass are selected. In comparing the colors we use a 3-slot comparator with a ground glass background illuminated by a daylight bulb. After 3 or 4 minutes the comparison with the standards may be started, but the final decision must be made

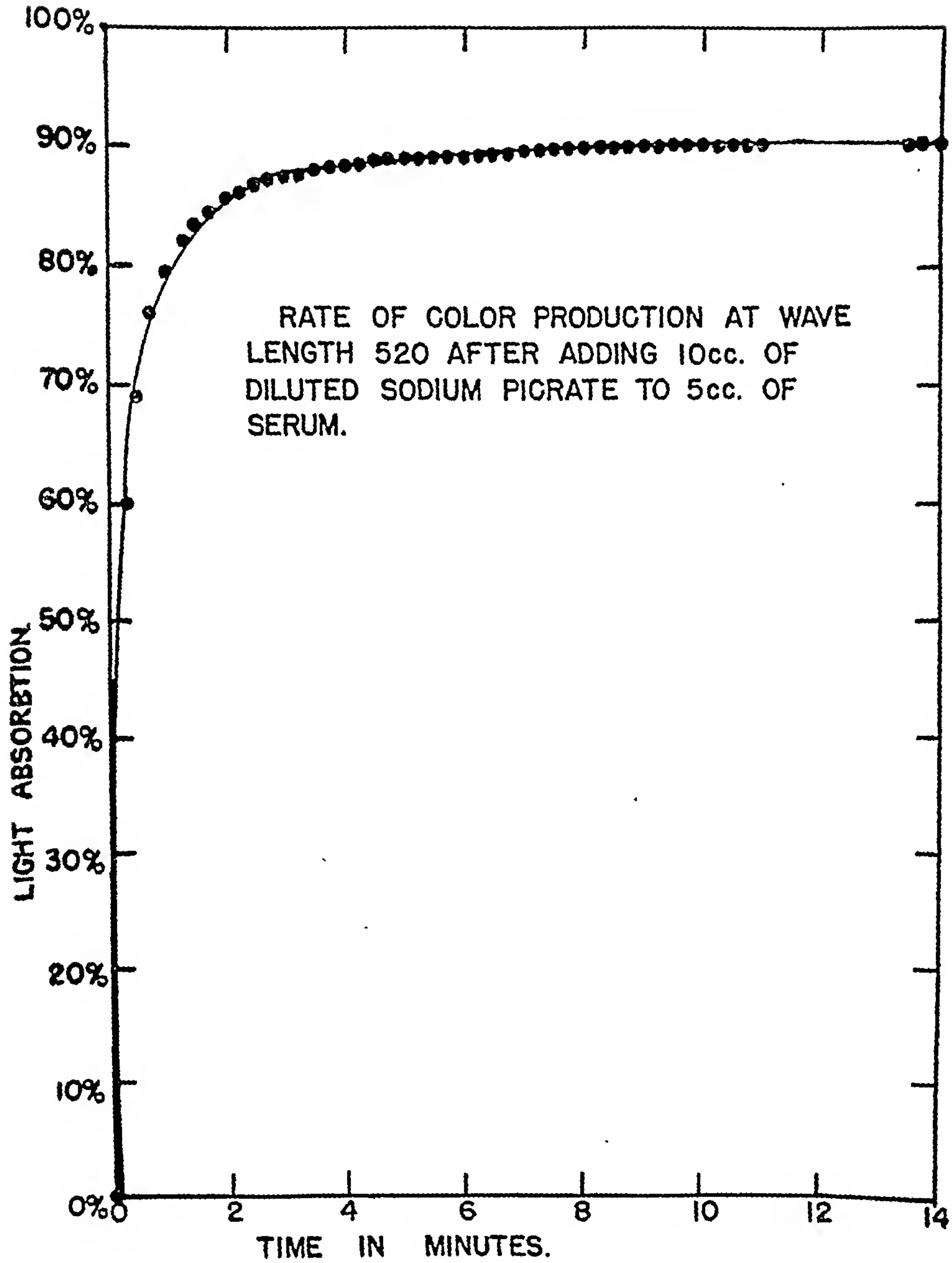

Fig. 1

when the bell rings 6 minutes after the picrate is added to the serum.

It is curious and fortunate that the colors of certain concentrations of potassium dichromate and of creatinine picrate in sodium picrate should be indistinguishable by eye and should give almost an identical light absorption when a 520 filter is used. It was potassium dichromate that Folin used as a standard in his first creatinine determinations (3). Not only do potassium dichromate solutions give a perfect match but, once evaporation is prevented, they remain unchanged for years. In Table I we list the quantities of potassium dichromate that, when dissolved in water and brought to a volume of 100 ml., give colors corresponding with the colors that develop in 6 minutes in sera containing the designated creatinine concentrations (mgm. creatinine per 100 ml. of serum).

Provided that the few measurements are made with care and granted that the user has the capacity to distinguish shades of yellow and orange, it has been found by many that this method is of value in the immediate management of new patients. It cannot give, of course, a continuous series of creatinine concentrations for classification, but it does afford grounds for the division of our pa-

TABLE I

Potassium dichromate concentrations (mgm. potassium dichromate dissolved in water and brought to 100-ml. volume)

These concentrations have colors that are equivalent to the colors obtained by the direct and filtrate clinical methods from sera containing the given concentrations of creatinine (mgm. creatinine per 100 ml. of serum).

Direct method		Filtrate method	
Potassium dichromate	Serum creatinine	Potassium dichromate	Serum creatinine
mgm. per 100 ml.		*mgm. per 100 ml.*	
200	0.47	50	0.27
250	0.64	100	0.57
300	0.80	200	1.14
350	0.98	300	1.65
400	1.15	400	2.10
450	1.31	500	2.49
500	1.48	600	2.94
600	1.80	700	3.36
700	2.10	800	3.81
800	2.40	900	4.17
900	2.69	1000	4.50
1000	2.96	2000	7.62
1500	4.15	3000	10.05
2000	5.24	4000	12.39
3000	7.10	5000	14.64
4000	8.62	6000	16.71
5000	9.88	7000	18.60
6000	10.97	8000	20.46
		9000	22.17

tients into 4 categories: those who may have concentrations within the normal range, and those with a slight, a moderate, or a pronounced elevation of concentration.

There are conditions in which the color of the serum is altered and then we cannot use the direct method. Bilirubinemia is an example, though one rarely seen in ambulatory patients with renal disease. Hemoglobinemia is more common, but it is almost invariably an artifact.[1] Lipemia constitutes our almost sole source of difficulty. The fat alters the quality of the color and when we try to read it against the dichromate tubes we find that we somewhat overestimate the creatinine concentration. As might have been anticipated, we failed to find any fat solvent, and we have tried many, that is able to clear these lipemic sera. In such cases, or when the serum is colored with bile pigment or hemoglobin, we can get water-clear filtrates by precipitating the color along with the serum proteins. We take 1.5 ml. of serum in a graduated centrifuge tube, add 3 ml. of 0.5 M. sodium acetate-acetic acid buffer at pH 5 to the 4.5 ml. mark, mix, close with a vaccine stopper through which a hypodermic needle is inserted, and place the tube in a beaker of boiling water for 5 minutes. The coagulum is then emptied onto a small filter and, when a litle more than 1 ml. of filtrate has run into a graduated centrifuge tube, the filter is removed and the volume reduced to exactly 1 ml. by a capillary pipette. Then we add 0.5 ml. of undiluted alkaline picrate (1 ml. of 10 per cent sodium hydrate plus 5 ml. of 1.175 per cent picric acid). The interval timer is set to ring at 10 minutes and the comparison is made with the dichromate standards given in Table I for the filtrate method.

This filtrate method was the one we first developed. It was conjoined with a similar method for the determination of the rate of creatinine excretion in the urine. Desiring a method requiring no more apparatus than graduated centrifuge tubes and capillary pipettes, and avoiding the need for color standards, we diluted the urine color until it matched the color of the serum and thus derived an approximate "creatinine clearance." However, by comparison with results obtained on the same serum and urine by precision methods, and through experiments on rats from which known proportions of the total renal tissue had been removed, we learned that, as a rule, a decrease in creatinine clearance arose not from any diminution in the rate of creatinine excretion but from an increase in the concentration of creatinine in the serum. So when we found that we were able to approximate the serum creatinine concentration without removing the protein and usually learn as much from it as we could from a clearance, we felt that we had probably reached that maximum of rapidity and ease of operation that is an indispensable prerequisite for a clinical as contrasted with a laboratory method.

It seems to us that this distinction between what we call laboratory and clinical methods necessarily involves a differentiation in the criteria by which they should be judged. A laboratory method belongs to science. It is an instrument for an exact description, under isolated and controlled experimental conditions, of what is measured. For this purpose a high order of average precision is ad-

[1] We draw the blood into a vaselined syringe. The needle is detached before transferring the blood to a centrifuge tube in which it is allowed to clot. When the blood is forced through the needle, some lysis of red cells may be induced.

vantageous. Often this precision requires a considerable complexity of mechanism. Occasionally a fault in one or other of the elements involved may lead to large error. The scientist accepts this possibility with equanimity. He knows that in the end such errors will reveal themselves as gross deviations from the general law he intends to derive from his measurements. A clinical method, on the other hand, is a doctor's tool. Reliability in each and every observation rather than average precision is its virtue. It is best, therefore, if the method has such a transparent simplicity that any error in its operation is at once apparent. The clinician is looking not for that which is general and abstract but for that which is individual and concrete. Every patient is for him an end. So he cannot afford to use a method, no matter how precise, that may at times mislead him with respect to the individual. What he wants is a universal dependability within known limits of error that need not be so very narrow.

We have evaluated this clinical method in terms of its greatest errors. In 93 patients the serum creatinine concentration was estimated by the standard Folin-Wu method, using the Evelyn colorimeter as well as by the direct addition of sodium picrate to serum (the clinical method). The series was divided into 4 groups: first, those in whom the serum creatinine concentration was not certainly abnormal, and the other 3 into those with slight, moderate, and pronounced increases in concentration. In Table II we give the maximum minus and plus errors of the direct clinical method for each group on the assumption that the photoelectric measurement gave us correct values.

In Table II we give the minus errors first because it is they that are dangerous. Even in these worst examples there is no case in which we were seriously misled. Always we were able to pick out the patients who had an increase clinically important to recognize. We believe, therefore, that this is a dependable method in the clinical sense, for in many hundreds of observations made since this series was completed there has been no single patient with serious renal failure that we have failed to detect by this simple procedure.

TABLE II

The largest errors incurred in the District Clinical Method as derived from comparison with determinations made by the precision method

Clinical subdivision	No. of patients	Minus errors	Plus errors
		mgm. per 100 ml.	
Possibly normal	63	0.6 instead of 1.2	2.1 instead of 1.1
Slight retention	15	3.0 instead of 3.3	3.0 instead of 2.3
Moderate retention	9	7.1 instead of 7.9	8.6 instead of 7.6
Marked retention	6	7.9 instead of 9.3	9.3 instead of 8.1

CONCLUSIONS

1. A clinical method designed to reveal the existence of varying levels of increase in serum creatinine concentration is described.

2. A modification of the method is given for use when the serum is lipemic or colored with bilirubin or hemoglobin.

BIBLIOGRAPHY

1. Addis, T., Barrett, E., Poo, L. J., and Yuen, D. W., The relation between the serum urea concentration and the protein consumption of normal individuals. J. Clin. Invest., 1947, 26, 869.
2. Barrett, Evalyn, and Addis, T., The serum creatinine concentration of normal individuals. J. Clin. Invest., 1947, 26, 875.
3. Folin, O., Beitrag zur Chemie des Kreatinins und Kreatins im Harne. Zeit. f. physiol. Chemie., 1904, 61, 223.

THE CORRELATION BETWEEN THE ALBUMIN AND ALPHA GLOBULIN CONTENTS OF PLASMA [1]

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The hypoalbuminemia which accompanies insufficient protein intake has come to be generally accepted as a diagnostic sign of protein deficiency (1). Experimental study of dogs depleted in protein by low protein diet feeding, manifests the same marked decrease in the plasma albumin, but not in the plasma globulin (2). Further study has shown this condition to be also characterized by an increase in the concentration of alpha globulins (3, 4). This loss of albumin, if expressed as total protein in circulation, becomes more apparent, since protein depletion is commonly accompanied by a decrease in plasma volume (4, 5). On the other hand, the total circulating alpha globulins remain essentially constant and other globulins decrease slightly. It would seem possible from these considerations that the decrease in the concentration of albumin and the increase in the concentration of alpha globulins might together furnish an index of the degree of protein depletion more valuable than that obtained from the albumin alone.

Were such a relationship to hold for man, as it would appear to hold for dogs under experimental conditions, then it would permit the utilization of the ratio of the two moieties of plasma protein as a measure of depletion.

The possibility has been examined by determining the concentration of plasma albumin and alpha globulins in the plasma of a number of patients with several types of diseases and with different levels of plasma protein concentration. The estimations were based upon data derived from electrophoretic patterns of plasma. A limited number of patients were also studied before, during, and after the administration of several protein hydrolysates to ascertain whether an increase of the percentage of plasma albumin is accompanied by a reciprocal change in the plasma alpha globulins. The results of these studies are the subject of this communication.

EXPERIMENTAL

Twenty ml. of blood from each patient under investigation were introduced into a centrifuge tube containing solid sodium oxalate as anti-coagulant. After centrifugation about 8 ml. of the plasma were diluted with an equal volume of diethylbarbiturate buffer of pH = 8.4 and ionic strength of 0.10 and then dialyzed against the same buffer for 24 hours or longer. After adequate dialysis the bag was opened and the solution was electrolyzed according to the technic of Longsworth (6). The electrolysis was carried out for a sufficient length of time (3 hours) to permit a clear-cut separation of alpha globulins from the albumin. The total plasma protein concentrations were determined by micro Kjeldahl.

RESULTS

One hundred ninety-eight samples of plasma taken from patients with several diseases were analyzed. The results are illustrated in Figure 1, in which per cent of plasma albumin is plotted against per cent of alpha globulins and a curve is fitted to the data. The distribution of the data about the mean is reasonably normal as indicated by the lines in the figure demonstrating the areas of 1 and 2 standard deviations. A correlation co-

[1] The author is indebted to Shirley DeBiase for her technical assistance in electrophoretic analyses.

TABLE I

The correlation between per cents of albumin and alpha globulins

Albumin *per cent*	Alpha globulins *per cent*								
	5–10	10–15	15–20	20–25	25–30	30–35	35–40	40–45	45–50
70–75	1	0	0	0	0	0	0	0	0
65–70	4	6	2	0	0	0	0	0	0
60–65	2	5	5	0	0	0	0	0	0
55–60	3	8	12	1	0	0	0	0	0
50–55	6	11	11	6	0	0	0	0	0
45–50	2	2	17	12	5	0	0	0	0
40–45	1	3	6	15	14	2	0	0	0
35–40	0	0	7	7	1	2	0	0	0
30–35	0	0	0	4	2	2	0	0	0
25–30	0	0	0	2	2	1	0	0	1
20–25	0	0	0	0	1	1	1	1	1
Total	19	35	60	47	25	8	1	1	2

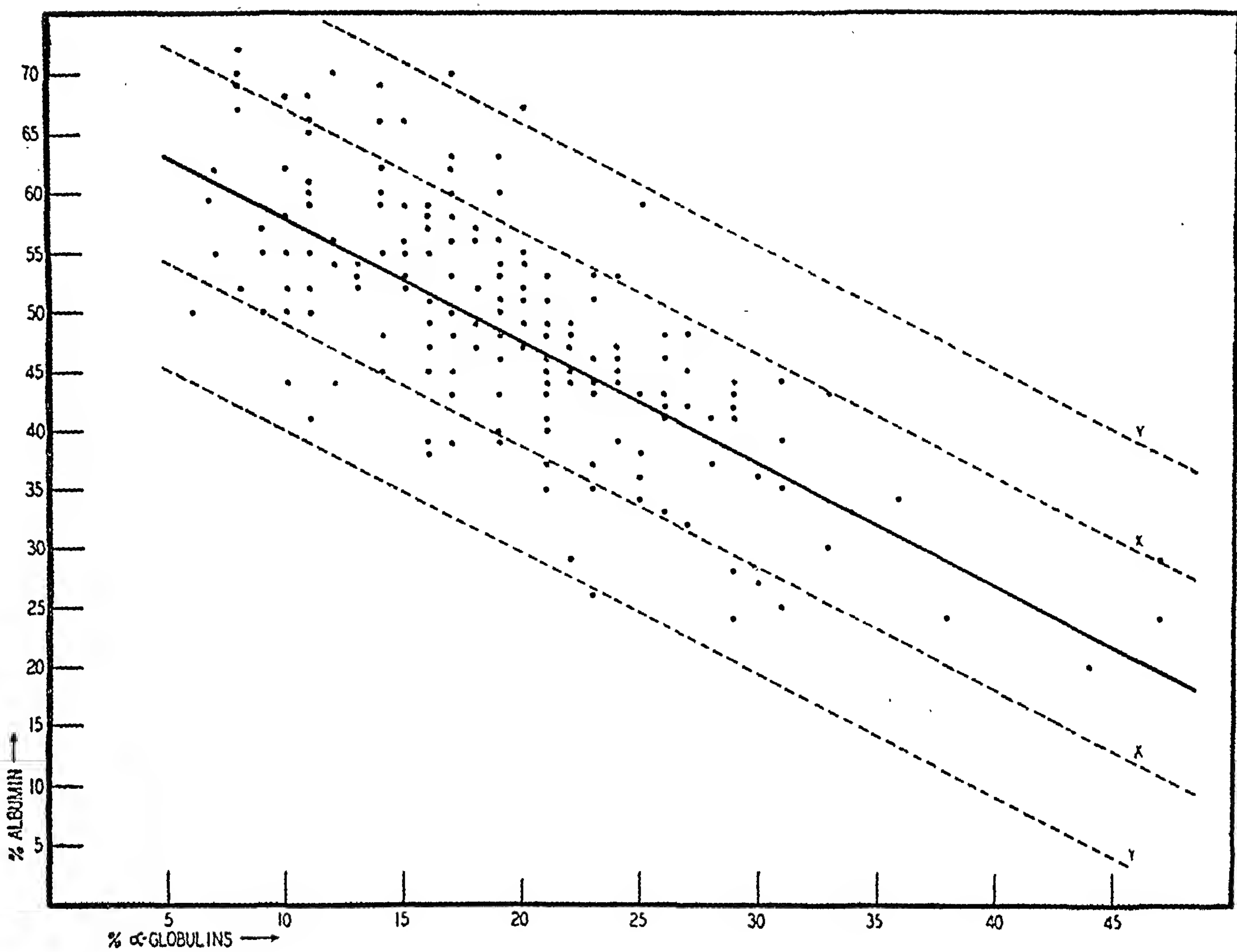

FIG. 1. THE RELATIONSHIP BETWEEN THE PERCENTAGES OF ALBUMIN AND ALPHA GLOBULINS

efficient (7) of −0.63 with a standard error of ±0.04 was also obtained by arranging the data according to the albumin levels grouped at intervals 5 per cent apart (Table I). A negative correlation coefficient of 0.4 or greater is commonly considered statistically significant; consequently the results demonstrate an inverse relationship between the concentrations of albumin and alpha globulins. However, the correlation is not sufficiently close to provide useful information when one considers a single sample of serum.

A similar attempt was made to establish a correlation between the per cent albumin and total plasma protein concentration. However, the data reveal no relationship with statistical significance. Twenty-five samples with total protein concentrations of 6.5 grams per cent or even higher, have albumin levels between 30 per cent and 40 per cent. At the same time, 33 samples of plasma with a protein concentration of 6.5 grams per cent or less have protein concentrations as high as 50 per cent or above. Thus, there appears to be no relationship between per cent albumin and plasma protein concentration. Unfortunately the plasma volumes of these patients are not available.

A casein hydrolysate [2] or other commercially available hydrolysates (at a dose of about 5 to 6 grams per kilogram body weight per day) were administered orally to 11 patients for several weeks. The first 8 patients (column I, Table II) were hypoalbuminemic. The 9th patient (Urban) had low protein concentration (4.8 gram per cent) but normal albumin content before treatment. The remaining 2 patients (Migut and Kjaer) had normal plasma protein concentration and normal albumin content.

The data do not constitute a comparison of the relative effectiveness of the various hydrolysates

[2] The casein hydrolysate was supplied by E. R. Squibb & Sons.

used. However, they do demonstrate that the administration of an adequate protein hydrolysate brings about plasma albumin regeneration despite differences in the state of hypoproteinemia. As is to be expected, this is most marked where the hypoalbuminemia is severe. Also, the increase of per cent of albumin is accompanied by a drop in alpha globulins. It is interesting to note that the albumin content of Summelensky was 39 per cent before treatment, was increased to 65 per cent after 3 weeks of treatment, but dropped to 43 per cent after 8 weeks of treatment. During the corresponding periods, the per cent of alpha globulins dropped when the albumin increased and increased when the albumin decreased.

The relationships between the concentrations of plasma albumin and alpha globulins in the second samples from these 11 patients were plotted in the same manner as the random samples in Figure 1. The relationships between these two moieties at different levels of protein concentration in the individual patient were generally closer than that of the random samples shown in Figure 1. This fortifies the view that an inverse relationship between the two usually exists, but again it is to be emphasized that the ratio of the two fractions is not useful in the evaluation of the nutritional status of any single patient. The compositions of the other plasma protein components are recorded in Table II, but the effects of administration of the hydrolysates on these components are erratic and no conclusion can be drawn.

TABLE II

The effect of administration of protein hydrolysate to hypoalbuminemic patients

Name	Hydrolysate given	Protein concentration gram per cent	Composition in per cent						Weeks of administration
			1	*2*	*3*	*4*	*5*	*6*	
Young	Squibb[1]	4.62	20	13	31	12	12	12	0
		6.52	37	9	13	16	10	15	2
			40	8	11	17	11	13	3
Egan	Amigen[1]	7.40	26	8	15	7	6	38	0
		7.68	40	6	13	9	5	27	4
Porter	Squibb	5.89	32	9	18	12	4	25	0
		8.0	45	4	13	9	4	25	
		8.5	50	4	2	10	13	21	
Lopez	Amigen	6.76	33	7	19	17	4	20	0
		6.40	52	7	6	9	10	16	6
Gates	Essamine[2]	5.99	35	9	14	7	13	22	0
		5.66	51	8	12	12	5	12	6
Summelensky	Squibb	6.02	39	9	15	10	7	20	0
		5.21	65	6	5	9	7	8	3
		6.01	43	10	19	14	1	13	8
Zacharchuk	Edamin[2]	6.36	41	15	11	14	4	15	0
		6.02	56	5	10	9	3	17	4
Davis	Squibb	5.36	43	10	15	12	4	16	0
		7.01	56	5	12	7	4	16	4
Urban	Squibb	4.84	55	11	4	15	8	7	0
		6.07	57	9	7	12	7	8	3
Migut	Squibb	8.00	48	9	17	10	3	13	0
		8.82	52	6	15	10	5	12	6
Kjaer	Squibb	7.32	59	6	19	8	2	6	0
		8.00	72	2	6	2	12	6	1

[1] Casein hydrolysates.
[2] Lactalbumin hydrolysates.

1 = Albumin
2 = Alpha$_1$ globulin
3 = Alpha$_2$ globulin
4 = Beta globulin
5 = Fibrinogen
6 = Gamma globulin

DISCUSSION

Electrophoretic analysis is a useful tool for the determination of the composition of plasma proteins. During the past few years, numerous investigations were made to correlate the change in plasma protein pattern with a specific disease. It has been found that diseases such as hyperthyroidism (8), malaria (9), pneumonia (10), streptococcus infections, relapsing fever, and scarlet fever (11) are accompanied by a rise in alpha globulins, some depression of albumin, and occasionally a rise in other globulins. These findings have usually been attributed to the disease rather than, as seems likely, the protein deficiency which is incidental to the disease.

The data presented include sera from 76 patients with peptic ulcer, 51 patients with tuberculosis, 25 patients with malnutrition, and 37 with miscellaneous conditions. These show that a lowering of albumin is generally accompanied by a rise in the alpha globulin. This increase, although statistically significant, is not sufficiently close for clinical use in the evaluation of the state of protein nutrition of the individual patient. The relationships are sufficiently similar to those observed in simple protein depletion to lead one to conclude that the alterations of normal plasma pattern are in fact due to the same fundamental mechanism, *i.e.*, inadequate protein retention as an incidental phenomenon in each of the diseases studied. It is not surprising then that the patients with a low albumin content of the plasma are treated by the administration of an adequate protein hydrolysate, thus correcting the protein deficiency; a rise of albumin occurs quite promptly and this is accom-

panied by a decrease in the concentration of alpha globulins.

SUMMARY

One hundred ninety-eight samples of plasma from patients with several diseases were analyzed electrophoretically. There appears to be a correlation between the decrease in albumin and the increase in the per cent of alpha globulins. The significance of this relationship with respect to diseases is discussed.

BIBLIOGRAPHY

1. Peters, J. P., Nitrogen metabolism in acute and chronic disease. Ann. N. Y. Acad. of Sci., 1946, 47, 327.
2. Weech, A. A., and Goettsch, E., Dietary protein and the regeneration of serum albumin. II. Comparison of the potency values of beef serum, beef muscle and casein. Bull. Johns Hopkins Hosp., 1938, 63, 181.
3. Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., Plasma protein metabolism—electrophoretic studies; chronic depletion of circulating proteins during low-protein feeding. J. Exper. Med., 1945, 82, 157.
4. Chow, B. F., Allison, J. B., Cole, W. H., and Seeley, R. D., Effect of protein depletion on plasma proteins in dog measured by electrophoretic analysis. Proc. Soc. Exp. Biol. & Med., 1945, 60, 14.
5. Weech, A. A., Wallstein, M., and Goettsch, E., Nutritional edema in the dog. V. Development of deficits in erythrocytes and hemoglobin on a diet deficient in proteins. J. Clin. Invest., 1937, 16, 719.
6. Longsworth, L. G., Recent advances in the study of proteins by electrophoresis. Chem. Rev., 1942, 30, 323.
7. Croxton, F. E., and Cowden, D. J., Applied General Statistics, 1940, p. 673.
8. Lewis, L. A., and McCullagh, E. P., Electrophoretic analysis of plasma proteins in hyperthyroidism and hypothyroidism. Am. J. Med. Sci., 1944, 208, 727.
9. Dole, V. P., and Emerson, K., Jr, Electrophoretic changes in plasma protein patterns of patients with relapsing malaria. J. Clin. Invest., 1945, 24, 644.
10. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., Electrophoretic patterns of normal and pathological human blood serum and plasma. J. Exper. Med., 1939, 70, 399.
11. Dole, V. P., Watson, R. F., and Rothbard, S., Electrophoretic changes in serum protein patterns of patients with scarlet fever and rheumatic fever. J. Clin. Invest., 1945, 24, 648.

BIOCHEMICAL AND HEMODYNAMIC CHANGES FOLLOWING THE SUBCUTANEOUS INJECTION OF GLUCOSE SOLUTION [1]

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It is well known that the loss of extracellular electrolyte may contribute to or even produce cardiovascular collapse. This is manifest in its most striking form in the crisis of Addison's disease (1), in diabetic acidosis and coma (2, 3) and in patients losing large amounts of fluid from the gastrointestinal tract (4, 5). In these conditions the losses of the sodium ion, the chloride ion, and water are often the primary and sole cause of circulatory collapse. The chain of events which ends in clinical shock following salt depletion has been experimentally studied (6, 7). Abstraction of salt from a healthy animal via the peritoneal route according to the Darrow-Yannet technique (8) produces hypotonicity of the extracellular fluid. Water then moves into the cells in response to osmotic forces. The consequent decrease in extracellular fluid volume occurs in both the interstitial fluid and the plasma. Diminution in the latter is readily detected by the rise in the hematocrit and in the concentration of the total serum protein. It is of particular significance that some of the circulating plasma protein is lost and that in terms of proportionate change, the plasma volume diminishes much more than does the extracellular fluid as a whole (7). These alterations in the composition of the fluid in the extracellular and intracellular compartments are an invariable accompaniment of salt depletion. If acutely induced, and of sufficient magnitude, the loss of salt results in a shock state akin to that seen following burns, trauma, or hemorrhage (9). The circulation slows and the venous pressure falls. These changes are associated with a diminished cardiac output (7). If untreated, death ensues.

It seems highly probable that during absorption of a glucose hypodermoclysis salt is removed from the circulation in a manner identical with that observed following the injection of fluid intraperitoneally. Moreover, it has been recognized clinically that the subcutaneous administration of non-electrolyte-containing fluid to certain patients may precipitate circulatory collapse and death. This has been noted in particular in patients with mercury poisoning and with diabetic coma (10, 3). Inasmuch as these conditions are known to be frequently associated with some measure of antecedent salt and water loss, it is possible that the shock state is precipitated by further salt depletion during glucose hypodermoclysis (11). Since the parenteral administration of glucose solutions to all types of patients, even those on the verge of cardiovascular collapse, is a commonly accepted procedure, the biochemical and hemodynamic changes which occur have been studied experimentally and are reported in this paper. It has been found that the subcutaneous administration of a sufficient volume of non-electrolyte-containing fluid usually produces salt depletion, and that this can result in peripheral vascular collapse.

MATERIALS AND METHODS

The effects of glucose solutions on circulatory efficiency have been studied in animals and in human patients in the post-absorptive state.

Two healthy female dogs weighing 7.96 and 10.86 kilograms were given 5 per cent glucose solution by subcutaneous injection, 720 ml. and 1,000 ml. respectively, during a 10- to 15-minute period. In the first experiment, measurements were made prior to and at 2.0 hours after the injection of the glucose solution. In the second, observations of cardiovascular function were made at intervals during 2.5 hours. These included determination of the direct arterial blood pressure, the circulation time, the cardiac output, and the volume of urine elaborated. The methods and the calculations employed have been described in a previous paper together with the procedure employed for measurement of changes in the plasma volume, extracellular fluid volume, and the circulating plasma proteins (7). These involve the determination of the whole blood hematocrit and non-protein ni-

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[2] Dr. Winkler died June 26, 1947.

trogen, the concentration of serum protein, and the balance of serum chloride.

Glucose hypodermoclyses have also been given to 4 patients in amounts ranging from 32 ml. to 72 ml. per kilogram of body weight. In 2 of these subjects, and in one other, the effects of intravenous glucose solutions on circulatory efficiency have been determined in control experiments as well. The studies with patients differed from the animal experiments in that the parenteral fluids were administered over a period of several hours, and the cardiac output was not measured. In 2 of the experiments the nitrogen clearances were followed as well as the changes in effective renal plasma flow as measured by the clearance of para-amino hippurate (PAH)[3] (12).

RESULTS

At 2.0 and 2.5 hours, respectively, following the administration of glucose solution subcutaneously in dogs 110A and 55P, profound circulatory collapse was present. The circulation time rose to 12 and 15 seconds from initial values of 8 and 7 (Table I). The mean arterial blood pressure declined 25 and 35 mm. Hg, respectively. The cardiac output diminished to ⅓ or ½ of the predepletion values.[4] Urine elaboration ceased almost entirely.

The biochemical changes were equally definite. The concentration of chloride in extracellular fluid decreased by more than 10 meq. per liter. Hemoconcentration appeared as indicated by the increase in hematocrit and serum protein content; plasma volume diminished.

In patient L. M., equally striking biochemical and hemodynamic changes followed the subcutaneous administration of glucose solution (Table II). Hypochloremia, hemoconcentration, diminution of plasma volume, loss of circulating plasma protein, hypotension, and lengthening of the circulation time occurred. In this patient, therefore, the injection of glucose solution, 72 ml. per kgm. of body weight, produced unequivocal circulatory collapse which responded to treatment with plasma and physiological saline.

In patients C. M., W. O., and M. W. the biochemical changes at the end of 4.0 to 5.6 hours were less pronounced, but nonetheless definite. The hematocrit, the hemoglobin, and the serum protein concentration all rose. The concentration of serum chloride fell in 2 of these subjects and was unchanged in 1; the plasma volume fell in all 3 subjects; plasma protein left the circulation. The effects of these changes on circulatory efficiency were either minimal or absent. The circulation time was prolonged in only 1 of these subjects (patient C. M.). Nitrogen clearance declined temporarily in 1 of the 2 patients in whom it was measured (patient M. W.). Renal plasma flow as measured by sodium para-amino hippurate did not change significantly (patients W. O. and M. W.).

In the control experiments, on the other hand,

[3] Obtained through courtesy of the Medical Research Division of Sharp and Dohme, Incorporated.

[4] In dog 110A a preliminary determination of cardiac output was not done. If the mean value for cardiac index, as determined by us in a large series of normal dogs, of 5.45 ± 1.43 liters per minute per square meter is taken as the initial value (7), the final value represents a decrease in cardiac output of 56 per cent.

TABLE I

Effect of glucose hypodermoclysis on body fluids and the circulation in dogs: Changes in blood and serum analyses, hemodynamics, and plasma

Dog	Time from start of experiment	Weight	Hypodermoclysis* 5 per cent glucose	Urine vol.	Serum† Cl	Serum† Total protein	Blood Relative cell vol.	Blood Hemoglobin	Circulation time	Mean arterial pressure	Oxygen Consumption	Oxygen A−V difference	Cardiac index	Change in Plasma volume	Change in Circulating plasma protein
	hours	*kgm.*	*ml.*	*ml.*	*meq. per liter*	*grams per cent*	*per cent cells*	*grams per cent*	*sec.*	*mm. Hg*	*ml. per min.*	*volume per cent*	*liters per min. per sq. meter*	*ml.*	*grams*
110A	0	7.96			104.0	7.26	37.8	10.1	8	125					
	2		720	12	93.9	8.16	43.3	11.0	12	100	85	8.8	2.42	− 71	−2
55P	0	10.86			105.2	5.59	30.8	8.2	7	135	118	3.3	5.94		
	2.5	11.64	1000	10	89.9	7.81	40.8	10.2	15	100	66	8.0	1.67	−186	−1

* Given immediately after initial determinations were made at start of experiments.
† Water content of serum (W_s) calculated from the serum total protein concentration (P_s) by the formula:

$$W_s = 99.30 - 0.889P_s \text{ (15).}$$

TABLE II

Effects of glucose hypodermoclysis on body fluids and circulation of 4 human subjects

Exp.	Subject† and dose of 5 per cent glucose	Time from start of experiment	Weight	Intake: Oral H_2O	Intake: Sub-cut. 5 per cent glucose	Intake: Intravenous	Urine: Volume	Urine: Cl	Serum*: Cl	Serum*: CO_2	Serum*: Total protein	Blood: Relative cell volume	Blood: Hemoglobin	Circulation time	Blood pressure	Clearance of: PAH	Clearance of: NPN	Change in: Plasma volume	Change in: Circulating plasma protein
	ml. per kgm.	*hours*	*kgm.*	*ml.*	*ml.*	*ml.*	*ml.*	*meq.*	*meq. per liter*	*meq. per liter*	*grams per cent*	*per cent cells*	*grams per cent*	*sec.*	*mm.Hg*	*ml. per min.*	*ml. per min.*	*ml.*	*grams*
1	L. M.	0	37.6						101.9	24.7	6.52	33.8	12.5	12	105/65				
	72	4.3	39.1	0	2700		790	40	87.8		7.93	45.8	15.6	15	90/64			−595	−23
		20.0	38.8	1400		2100	?	?	96.9	23.2	7.21	36.8	13.6	12	90/60			+383	+19
2	C. M.	0	72.6						94.3	29.5	6.95	46.0	16.2	16	114/60				
	39	5.6		0	2850		555	26	92.5	29.4	7.21	49.0	16.6	22	124/76			−270	−11
		24.0	71.3	1200			1700	21	94.0	29.6	6.80	47.3	15.6	16				+320	+10
3	W. O.	0							98.9	25.4	6.66	47.8	16.4	13	118/74				
	41	0.5				70‡	221									746	28.5		
		4.0		0	2700	200‡	710	6	90.3	26.6	7.16	54.0	17.3			578	28.3	−490	−20
		4.5	66.6			30‡	62							12	116/64	752	25.4		
4	M. W.	0	62.2						97.7	27.6	7.16	45.2	15.0	13	128/88				
	32	0.5				80‡	217									669	31.3		
		4.0		0	2000		620	18	92.3	25.7	8.74	54.0	17.0	12	130/90		19.0	−730	−20
		4.3	63.2			40‡	42									561	35.0		

* Water content of serum (W_s) calculated from the serum total protein concentration (P_s) by the formula:

$$W_s = 98.5 - 0.745P_s \ (16).$$

Balance data are expressed per individual period rather than cumulatively. The hypodermoclyses were given at start of experiments. Time from start of experiment indicates end of period at which time serum analyses and hemodynamic measurements were made.

† L. M.: 33-year-old white female, ? tuberculosis of abdominal lymph nodes.
C. M.: 44-year-old white male, tertiary cerebrospinal lues, penicillin treatment.
W. O.: 27-year-old white male, idiopathic epilepsy.
M. W.: 26-year-old negro male, latent lues, penicillin treatment.

‡ PAH in 5 per cent glucose solution.

TABLE III

Control experiments: effects of glucose infusions on body fluids and circulation of 3 human subjects

Exp.	Subject** and dose of 5 per cent glucose	Time from start of experiment	Weight	Intake: Oral H_2O	Intake: Intravenous 5 per cent glucose	Urine: Vol.	Urine: Cl	Serum*: Cl	Serum*: CO_2	Serum*: Total protein	Blood: Relative cell volume	Blood: Hemoglobin	Circulation time	Blood pressure	Change in: Plasma volume	Change in: Circulating plasma protein
	ml. per kgm.	*hours*	*kgm.*	*ml.*	*ml.*	*ml.*	*meq.*	*meq. per liter*	*meq. per liter*	*grams per cent*	*per cent cells*	*grams per cent*	*sec.*	*mm.Hg*	*ml.*	*grams*
5	L. M.	0	36.9					96.9		6.47	33.8	12.5	11	100/70		
	70	5.6	37.4	400	2600	1960	30	98.0		6.64	34.0	12.5	11	110/78	− 8	+ 2
		23.0	37.0	1055		?		103.6	24.3	7.33	38.7	12.7	11		−144	+ 1
6	C. M.	0	71.3					94.0	29.6	6.80	47.3	15.6	16			
	42	5.5	72.4	0	3000	1140	12	97.1	28.2	6.19	43.8	15.6	14		+230	− 7
		24.0	70.4	2400		2970	91	95.9	28.4	6.19	41.1	15.0			+320	+20
7	D. P.	0	77.8					99.9	23.2	7.09	40.7	13.9		116/70		
	39	5.0	77.8	200	5000	2500	42	102.2	24.0	7.01	40.8	13.9		122/70	− 10	− 3
		24.0	77.9	ad. lib.		1475	73	97.1	24.2	7.43	39.7	14.1		132/78	+ 20	+16

* See footnote to Table II.

Balance data are expressed per individual period. Infusions were given at start of experiments. Serum analyses and hemodynamic measurements are as indicated in Table II.

** L. M. and C. M.: See Table II. D. P.: 65-year-old white male, benign gastric ulcer, subsequent subtotal gastrectomy.

administration of comparable amounts of glucose solution intravenously failed to induce hypochloremia, hemoconcentration, and loss of circulating plasma protein, or to affect adversely the circulatory dynamics (Table III). Urine volumes were maintained, or actually increased, throughout the periods of observation.

DISCUSSION

These studies with animal and human subjects provide unequivocal proof that the subcutaneous injection of fluid which does not contain electrolyte may produce a form of salt depletion. Even though the electrolytes which diffuse into the subcutaneous pool of fluid are still within the body as a whole, they have been removed from the circulatory system and from the usual confines of interstitial fluid. Such segregation depletes the rest of the body of salt. This can produce circulatory collapse.

In the studies reported here, definite impairment of circulatory efficiency occurred in the dog experiments and in 1 of the 4 experiments with humans. Salt depletion definitely was present, however, in 5 of the 6 subjects given glucose solution by subcutaneous injection. Hence, in the patients who failed to develop evidences of cardiovascular collapse the salt depletion was either of insufficient magnitude or only a transient phenomenon. The 2 dogs received 92 ml. and 90 ml. of glucose solution per kgm. of body weight. If none of the injected fluid had been absorbed and if chloride had diffused into it to the point of equilibrium with the body fluids, 32 and 33 per cent respectively of the total body chloride would have been segregated.[5] On the other hand, even though all 4 patients received approximately 3 liters of fluid subcutaneously at comparable rates of flow, the per kilogram doses varied widely. Patient L. M. who developed shock received 72 ml. per kgm.; 33 per cent of the chloride was thereby removed from the rest of the extracellular fluid. The other 3 patients were given only 32 to 41 ml. per kgm. As a consequence, a distinctly smaller amount of body chloride, 16 to 19 per cent, was segregated in the subcutaneous pool. This was enough to produce the typical biochemical changes but not sufficiently great to result in cardiovascular collapse. These are apparently the limits of the changes in most patients, since a glucose hypodermoclysis is usually a benign procedure. Under these circumstances the abstraction of body electrolytes is both minimal and temporary, since the salt is returned to the extracellular fluid as a whole as the clysis is absorbed. This salt depletion may, however, be augmented and cardiovascular collapse produced, as evidenced in the dog experiments and in 1 of the 4 studies with human subjects, by increasing the relative volume of the glucose solution.

Shock may develop, however, even after a comparatively small hypodermoclysis. This is particularly true in patients who already have some measure of salt depletion as a consequence of their illness, and in those who are on the verge of circulatory collapse because of trauma, burns, etc. In these patients even a relatively small loss of extracellular electrolyte may plunge them into manifest shock. This has been noted clinically and reported (3, 10). In patients such as these, with the efficiency of the circulation already partially compromised, subcutaneous fluid is poorly absorbed and the salt depletion ordinarily produced by small volumes of fluid is magnified and prolonged. Shock is, therefore, not only precipitated, but perpetuated as well.

It cannot be assumed, therefore, that glucose solution can be administered subcutaneously with impunity to all patients. Particular care must be exerted toward the identification of patients in imminent circulatory collapse, with or without salt depletion. In subjects such as these it is imperative that the subcutaneous administration of glucose solution be either preceded or accompanied by measures to prevent or combat shock. It should be an invariable rule that, whenever subcutaneous parenteral fluid is to be administered, the saline fluids precede salt-free glucose solutions. If incipient shock is suspected the patient should be treated with blood, plasma, or gelatin and by res-

[5] Undoubtedly these assumed conditions were not realized; the degree of absorption of the fluid and its chloride concentrations were not measurable. But the values for chloride segregation obtained on these assumptions indicate maximum limits and are valid for comparison between experimental subjects. The initial extracellular fluid volume is also assumed to be 25 per cent of the body weight in dogs and 20 per cent of the body weight in humans.

toration of salt deficits prior to the use of subcutaneous glucose.

Experimental studies provide a logical basis for therapy of patients in whom shock has developed through the inadvertent use of glucose solution subcutaneously. It has been shown that, within certain limits of time, shock produced by acute salt depletion can be completely, or almost completely, reversed by the simple replacement of the salt deficit with hypertonic solutions of saline (13). It has been found, however, that the use of colloid solutions together with replacement of salt provides a distinct margin of safety (14). Hence blood or plasma should be administered in addition to salt solutions in treating salt depletion shock.

The lack of adverse effects following intravenous glucose solutions in the control experiments in Table III does not indicate that this is invariably a safe procedure. The patients studied had no demonstrable disorder of the cardiovascular-renal system and were not salt-depleted. The extra fluid merely increased the volume of urine. No data are available to indicate whether such a procedure would be equally benign in patients with antecedent salt depletion, on the verge of circulatory collapse, and unable to excrete water.

SUMMARY AND CONCLUSIONS

1. Five per cent glucose solution was administered by subcutaneous injection to dogs and to human subjects. The changes induced in the body fluids and circulatory dynamics were studied.

2. The subcutaneous injection of glucose solution usually produced transient salt depletion.

3. Large or rapidly administered hypodermoclyses of glucose solutions resulted in marked salt depletion and circulatory collapse.

4. It seems probable that circulatory collapse may be precipitated even with small glucose hypodermoclyses in patients with incipient shock.

BIBLIOGRAPHY

1. Loeb, R. F., Effect of sodium chloride in treatment of a patient with Addison's disease. Proc. Exp. Biol. & Med., 1933, 30, 808.
2. Atchley, D. W., Loeb, R. F., Richards, D. W., Jr., Benedict, E. M., and Driscoll, M. E., On diabetic acidosis; a detailed study of electrolyte balances following withdrawal and reestablishment of insulin therapy. J. Clin. Invest., 1933, 12, 297.
3. Danowski, T. S., Winkler, A. W., and Peters, J. P., Salt depletion, peripheral vascular collapse, and the treatment of diabetic acidosis. Yale J. Biol. & Med., 1946, 18, 405.
4. Gamble, J. L., Extracellular fluid; extracellular fluid and its vicissitudes. Bull. Johns Hopkins Hosp., 1937, 61, 151.
5. Peters, J. P., Symposium on fluid and electrolyte needs of surgical patient; the structure of the blood in relation to surgical problems. Ann. Surg., 1940, 112, 490.
6. Nadal, J. W., Pedersen, S., and Maddock, W. G., A comparison between dehydration from salt loss and from water deprivation. J. Clin. Invest., 1941, 20, 691.
7. Elkinton, J. R., Danowski, T. S., and Winkler, A. W., Hemodynamic changes in salt depletion and in dehydration. J. Clin. Invest., 1946, 25, 120.
8. Darrow, D. C., and Yannet, H., Metabolic studies of the changes in body electrolyte and distribution of body water induced experimentally by deficit of extracellular electrolyte. J. Clin. Invest., 1936, 15, 419.
9. Blalock, A., Principles of Surgical Care: Shock and Other Problems. C. V. Mosby Co., St. Louis, 1940.
10. Peters, J. P., Eisenman, A. J., and Kydd, D. M., Mercury poisoning. Am. J. M. Sci., 1933, 185, 149.
11. Elkinton, J. R., Danowski, T. S., and Winkler, A. W., The etiology and significance of hypochloremia (unpublished).
12. Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease. The Commonwealth Fund, New York, 1944.
13. Danowski, T. S., Winkler, A. W., and Elkinton, J. R., The treatment of shock due to salt depletion; comparison of the hemodynamic effects of isotonic saline, of hypertonic saline, and of isotonic glucose solutions. J. Clin. Invest., 1946, 25, 130.
14. Winkler, A. W., Danowski, T. S., and Elkinton, J. R., The rôle of colloid and of saline in the treatment of shock. J. Clin. Invest., 1946, 25, 220.
15. Danowski, T. S., Elkinton, J. R., and Winkler, A. W., The deleterious effect in dogs of a dry protein ration. J. Clin. Invest., 1944, 23, 816.
16. Eisenman, A. J., Mackenzie, L. B., and Peters, J. P., Protein and water of serum and cells of human blood, with a note on the measurement of red blood cell volume. J. Biol. Chem., 1936, 116, 33.

ON THE CONCENTRATION OF PROTEIN IN SAMPLES OF NORMAL URINE MEASURED BY ITS SURFACE ACTIVITY

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INTRODUCTION

The appearance of albumin in quantity sufficient to give "positive" results by well known "clinical" precipitation tests, has long been regarded as a reliable indication of kidney dysfunction. The reliability, of course, depends on the assumption that in normal physiology, the glomerular "filter" is wholly impermeable to protein molecules. Direct evidence of this came from the beautiful experiments of Richards and his co-workers. Walker, *et al.* (1) found that in some of their samples of capsular fluid in the mammalian kidney the protein was less than 30 mgm. per cent, although the smallest degree of mechanical trauma resulted in the appearance of large amounts of protein. The question of resorption of protein in the tubules has been raised, though Bott and Richards found no evidence of this in amphibia (2). Some textbooks state that protein is not a normal constituent of urine (3). Other textbooks are content to state that "normal urine usually contains a trace of albumin too small to be detected by regular tests" (4). Again a "faint trace" by these tests is variously estimated from 30 mgm. per cent to as much as 100 mgm. per cent of protein (5a and b), and quantitative information as to the amount of protein found in normal urine is hard to find in the literature. Even though there have been discussion and work on the so-called "normal" or "benign" albuminurias (6a and b), quantitative measurements even in this field are lacking.

Bodansky (7) states, without reference, that normal urine contains 2 to 5 mgm. of protein per cent; Kolmer and Boerner (8) give figures that indicate an average of 5 mgm. per cent, and Everett (5) says that normal urine contains 1 to 6 mgm. per cent. Most references are to the classical work of Mörner (9), who found from 2.2 to 7.8 mgm. per cent protein. However, he was more concerned with the chemical nature of the protein and related substances than with a statistical evaluation of the quantity normally present.

The quantitative methods of measurement of protein in urine are capable of measuring much smaller amounts, down to perhaps 5 or 10 mgm. per cent but this is only if relatively large samples are available. Thus, they are not suitable for quantitative studies on the time-course of albuminuria, produced experimentally or pathologically.

It is evident that there is a possibility that a given sample of urine might contain protein in amounts too small to be detected by the commonly used methods, yet this amount could be much greater than the amount found in truly "normal" urine. It becomes of importance to establish in a quantitative and statistical manner, what amount of protein is to be expected in a sample of normal urine. The method to be described is an adaptation of one first demonstrated to us by Dr. J. B. Bateman, for measuring the amount of protein in cerebrospinal fluid, and so used by Bazett and Burton (10). It is essentially the method of Gorter and Grendel (11), for serum proteins, simplified for clinical use and applied to urine.

APPARATUS

The apparatus required is very simple and inexpensive. (See Figure 1.) Surface films are formed on buffer in a plastic tray (of lucite) 36 by 11.5 by 2 cm. over-all, with a well which is accurately 10 cm. wide and 0.5 cm. deep. Black plastic is used for the bottom of the tray, as this greatly facilitates seeing the oil-drops used in the method. Several "barriers" of plastic, 12 cm. long and ¼ inch square in cross section, are required, and one of these is rigidly fixed at right angles to a long guide piece, carrying a scale which shows the area of the well enclosed between the barrier and the end of the tray. All other apparatus (a 5-ml. burette, pipettes, etc.) is standard in chemical laboratories, except perhaps the platinum wire set in a glass handle. The total cost of apparatus is certainly less than $10.00.

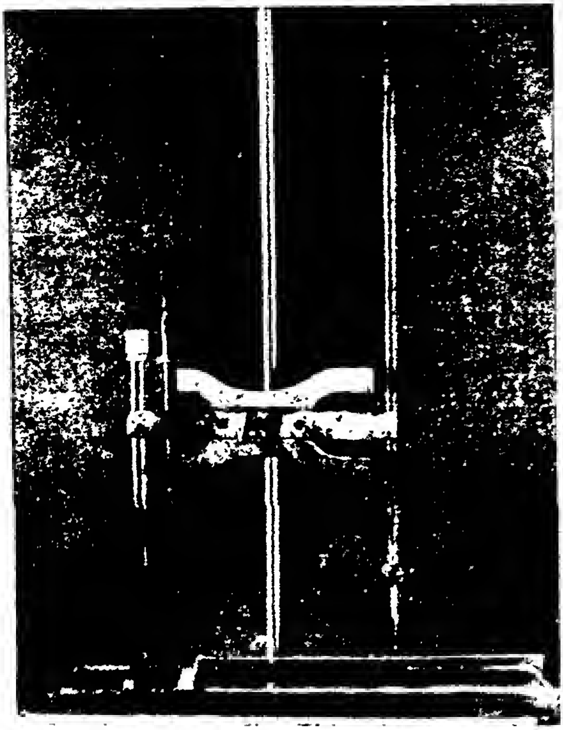

FIG. 1

METHOD

The method consists of measuring the total surface activity of a sample of urine and then the residual activity after protein has been precipitated. The difference gives the activity due to protein. This divides itself naturally into several successive steps:

(*a*) *Preparation of trays and barriers*

The tray and barrier must first be cleaned with distilled water and rendered hydrophobic. We have found that paraffin wax is much less satisfactory as a hydrophobic agent than is ferric stearate. This, though difficult to purchase, is very easily made. The following instructions are given by Dr. A. DeLuca who kindly made the ferric stearate we used:

To some hot water (80° C.) add a quantity of stearic acid. The latter melts forming a layer above the water. Next, add a solution of $FeCl_3$ drop by drop until the water layer is a deep brown. Continue the heating for 15 to 30 minutes with stirring. Let the mixture cool, after which the ferric stearate forms a solid sheet above the liquid. Wash the impure compound by adding it to hot distilled water, and again allowing it to cool. The ferric stearate which reforms on cooling is then broken into small pieces and dropped into a test tube. The latter is placed in a water bath to melt the compound. After cooling the ferric stearate may be removed from the test tube in the form of a solid stick. It may be necessary to pass the test tube momentarily through a flame in order to cause the stick to fall out readily.

All surfaces that will be in contact with the liquids are rubbed with a stick of ferric stearate, then rubbed to a hard polish with paper tissue (Kleenex). This surfacing with stearate should be repeated at frequent intervals, certainly after each day of use of the apparatus.

(*b*) *Buffer used*

A buffer of pH 6.4 (307 ml. of 0.1 M citric acid added to 693 ml. of 0.2 M disodium phosphate) has been routinely used. This pH was chosen as the result of experiments on the surface activity of a given urine sample on buffers of various pH values. The method is most satisfactory from pH 6.0 to pH 7.0. The buffer is poured into the tray until it stands well above the edges, the shape of the meniscus indicating the "unwettable" nature of the stearate-treated plastic material.

(*c*) *Removing contamination*

Contamination by surface-active substances either from the tray and buffers or from the air is inevitable. It is minimized by "sweeping" the surface of the buffer with the barriers repeatedly and wiping the barriers clean between each operation with a fresh piece of paper tissue. This "sweeping" was first discovered by Pockels (12). We routinely have swept the tray 3 times before each "blank" or urine determination.

(*d*) *Forming the protein monofilm*

Protein is so active in forming surface films that for the range of concentration of interest in the research, namely from 0 to 80 mgm. per cent, the sample of urine is first diluted in the ratio of 10 to 1 with distilled water. (One ml. urine was added to 9 ml. of distilled water.) The diluted sample was then placed in the 5-ml. burette. The stop-cock of the burette should have a minimal amount of stop-cock grease, which is itself surface-active. Unless solutions containing organic solvents are left for long periods of time in the burrette, we have found little trouble from contamination with stop-cock grease. Where there is trouble from this source, it can be avoided altogether by using a dropping-pipette having no stop-cock.

To form the protein film, all that is needed is to allow the diluted urine in the burette to drop slowly (at the rate of 1 drop every 2 or 3 seconds) from a small height into the surface of the buffer (method of Gorter and Grendel [11, 13]). The top of the burette should be just high enough so that the drops do not touch the surface but fall on to it. Any surface-active substance tends to form a "skin" on the surface of the drops and when these reach the flat surface of liquid in the tray the material spreads rapidly. It will be noted that the drops persist for 2 or 3 seconds on the top of the buffer before they [illegible]

(e) Measuring the area covered by the film

A barrier pushed along the tray will carry the protein film before it and, after all available space on the surface of the buffer has been occupied by the film, further compression will raise the film pressure as the area decreases. The measurement of area has meaning only if it be made at a definite pressure. To indicate the pressure, the simple device is used of an indicating oil drop, as developed by Blodgett (14), Langmuir and Schaeffer (15). The platinum wire is cleaned by holding in a flame, is then dipped in a test tube of lubricating oil (crank-case oil), and touched briefly to the surface containing the protein film. The oil spreads to a small colorless disc which should not be more than 1 cm. in diameter. As the barrier is pushed up to diminish the area of the film, the area of the oil disc also decreases and its thickness increases, until it is thick enough to show interference colors. These are seen much better in diffuse daylight than in artificial light. The first tint is a straw color, followed with more compression by a first-order blue color, then complicated colors of higher order follow in quick succession as the thickness of oil increases. The oil will always reach a definite thickness, and show a characteristic interference color, at a definite film pressure. We have found that the first-order blue color gives the best critical endpoint. The barrier is therefore pushed steadily along the tray until this color is seen in the oil. Calibration of the particular oil used must be made on a surface balance, of the type now well known, to find the film-pressure corresponding to the endpoint used. In the case of our oil this is 9 dynes per cm. By mixing some "oxidized" (dirty) [1] oil with clean oil, indicators can be obtained that show critical colors at pressures from 5 to 15 dynes per cm. It will be seen that for the purpose of measuring protein in urine, it is not necessary to know the exact critical pressure of the oil used, except within wide limits, since calibration on standard protein solutions is employed. However, if the areas of film are measured under a different pressure, the calibration curve of the method will, of course, be different from that found in our case.

It will be found that when the endpoint of the critical color is first reached, the color will fade and the area will have to be reduced slightly to produce a permanent indication of endpoint. This is due to viscous properties in the elasticity of the protein film, and the endpoint used in the measurement is that which persists for at least 30 seconds without further movement of the barrier. The area, at this critical point, is then read from the scale.

Since the measurement is made at a film-pressure of 9 dynes per cm., the measured area is considerably less than it would be if made at "zero" pressure, *i.e.* than the limiting area at zero compression. If the amount of protein dropped on to the surface is too great, the total available area of the tray may be inadequate to accommodate it, and the later drops will not be able to form a monomolecular film. It is important, therefore, to ensure that when the film is formed, the area available is adequate. It was found by repeated tests that if the final area, measured under 9 dynes per cm. pressure, was less than half the total area of the tray, the area was proportional to the volume of diluted urine put on the tray, but if the final area were greater, the measured area was less than would be expected from the amount put on (Figure 2). This indicates that the limiting area (at zero pressure) is about twice that at the pressure of 9 dynes per cm. used in the measurement. A rule was therefore made that if the measured area was greater than half the area of the tray (say with 1 ml. of urine), the determination was repeated using a smaller quantity (say 0.6 ml. of urine), until the final area was sufficiently small. Alternately, the urine may be further diluted (say to 20 to 1 instead of 10 to 1) and the 1 ml. used as previously.

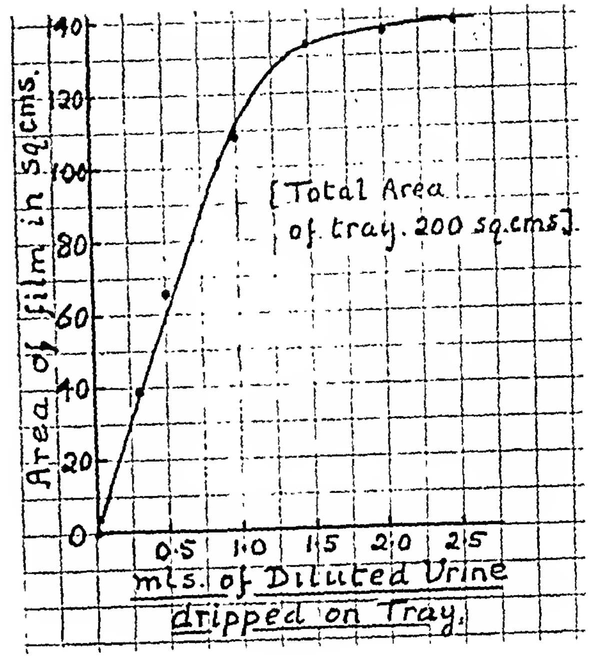

FIG. 2

(f) Precipitation of the protein

Several of the well-known precipitation methods were tried before one was found that was satisfactory. Some, such as trichloracetic acid, while precipitating the protein will markedly increase the residual activity of the non-protein surface-active substances in urine, probably by the release of more active groups. Other reagents, such as tungstic acid and lead acetate, produce a copious flocculent precipitate, and the residual surface activity is greatly reduced, presumably by the absorption of all surface-active substances on the precipitate. Finally, it was found that Tsuchiya's [2] reagent was satisfactory.

[1] "Oxidized" oil can be easily produced by heating clean oil in an open dish for a few minutes.

[2] Tsuchiya's reagent is: Phosphotunstic acid — 1.5 grams
Concentrated HCL — 5.0 ml.
95 per cent ethyl alcohol — 93.5 ml.

The activity of urine, freed from protein by precipitation by the reagent, was measured, a considerable amount of egg albumin added to increase greatly the measured activity; the added albumin was precipitated again by the reagent, the pH adjusted to neutrality with ammonia, and the activity finally measured. After correction for the dilution due to the added reagent, the final activity was consistently about the same as at the start. The success of this reagent is probably due to the use of alcohol which produces a compact precipitate of small surface area, and the absorption of surface-active substances on the precipitate is correspondingly low. It will be seen that in most cases, for clinical determinations, the precipitation and second measurement of activity after precipitation may be omitted.

(g) Routine of measurements

The sequence of measurements on the tray is illustrated by the table below (Table I) which is for a typical sample of normal urine diluted 10 to 1. It will be seen that "blank determinations" are made between each "test" measurement. In these the tray is "swept" 3 times, but nothing is added from the burette before the area of film is measured. The areas of "blanks" represent the inevitable contamination. The mean of the "blank" determinations is subtracted from the mean of "test" values to give the area due to the substances added from the burette.

TABLE 1

Urine sample diluted 10 times

Before precipitation			After precipitation		
Ml. added	Cm. on scale	Corrected values	Ml. added	Cm. on scale	Corrected values
0	1.3		0	1.4	
1.0	9.7	8.4	1.0	2.9	1.5
0	1.2		0	1.5	
1.0	9.5	8.2	1.0	3.1	1.7
0	1.4		0	1.2	
Mean		8.3			1.6 cm.

Area per ml. of urine = 830 (8.3 × width of tray × dil.) = 160 sq. cm.

Area due to protein = 830 − 160 = 670 sq. cm. in 1 ml. of original urine.

The whole procedure can easily be completed in 20 minutes.

CALIBRATION OF THE METHOD

Calibration was made by measurement of the surface area, due to protein, of a number of samples of urine containing increasing amounts of protein, the amount of protein being also measured by the quantitative gravimetric method of Folin and Denis (16). It was felt that in the calibration, urinary protein, rather than any other albumin, should be used. The urine of a patient, which was found, by the gravimetric determination, to contain

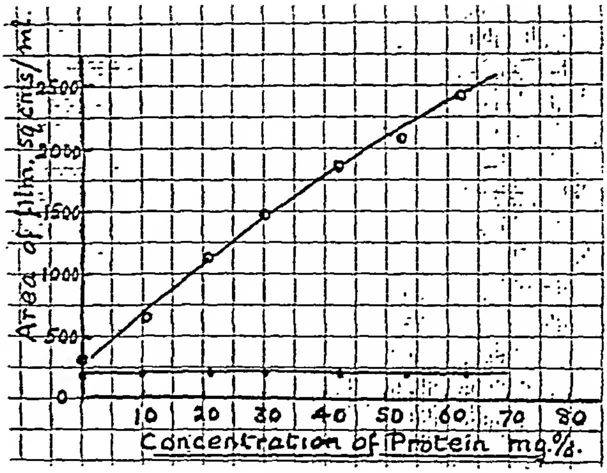

FIG. 3

240 mgm. protein per cent was, therefore, after neutralization, added in different proportions to normal urine which had been rendered free from protein by precipitation. The amount of protein in each of the samples was calculated from the proportions of a mixture of the normal and the protein-containing urines. Results are given in Figure 3, where the open circles represent surface area of film per ml. of original sample before, and the closed circles after, protein precipitation.

RESULTS

(a) The non-protein or "residual" surface activity in normal urine

The nature of the "residual" surface-active substances is the subject of a separate research now in progress. In normal urine, the residual activity, found after the protein has been precipitated, is a major part of the total surface activity. The substance or substances responsible for the residual activity are not yet identified, but their activity in normal urine is very constant. In 30 normal subjects, the residual activity from subject to subject, and in the same individual, had a mean from day to day of 206 sq. cm. per ml. of urine with a standard deviation of ±41 sq. cm. Reference to the calibration curve, Figure 3, shows that this amount of variability could correspond to a variation in protein of only ± 1 mgm. per cent. On the other hand, where there is an amount of protein greater than normal, even as little as 20 mgm. per cent, the residual activity is a small part of the total activity. Thus for most clinical purposes, the second step of precipitating the protein and remeasuring the surface activity (unless the presence of bile salts

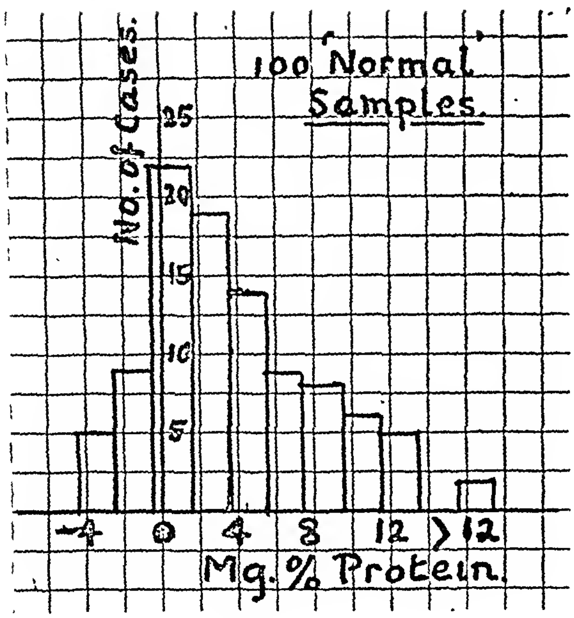

FIG. 4

is suspected), is unnecessary, especially if the total activity indicates a protein concentration within the normal range. To verify this, in 4 cases picked at random, the estimate of protein concentration from the total activity and from Figure 3, was compared with the value from the difference in activity before and after precipitation of protein. In no case did the 2 estimates differ by more than 3 mgm. per cent.

(*b*) *The concentration of protein in samples of normal urine*

Samples of urine were examined by the method described for 100 normal male subjects, most of them students of the Medical School, University of Western Ontario. The criterion of normality was that the blood pressure was within the normal range, with systolic pressure not greater than 140 mm. Hg and diastolic pressure less than 95 mm. Hg and that there was no known history of kidney dysfunction.

Results are shown in the histogram Figure 4. The negative values are to be expected because, as in all methods, there is an experimental error; and the true mean value is close to zero. A negative value means that the "residual" surface activity after precipitation was greater, instead of less, than the total activity before precipitation. In the case where the protein concentration is deduced from the total surface activity alone, without performing the precipitation, it means that the total activity of that particular sample fell below the "residual" activity on the standard curve.

The mean protein concentration is 3.7 mgm. per cent but it should be noted that the distribution is decidedly "skew," and the most frequently occurring concentration (the mode) in normals is between 0 and 2 mgm. per cent.

(*c*) *The concentration of protein in urine from patients with higher blood pressure than normal*

The amount of protein in the urine of normals turns out to be so very low that it seemed worthwhile to see if an appreciable number of patients with hypertension, but with no "clinical" evidence of proteinuria, would prove to have concentrations of protein significantly above normal, though below the limit of the normal clinical tests. The group of 50 patients from Victoria and from the Westminster Hospitals, London, Ontario, include 14 who had had some history of heart failure or some degree of decompensation but were free of such conditions at the time of this experiment. Most of the rest were diagnosed as "labile hypertensives," and the clinical tests had shown albumin in amounts described as a "very faint trace" or "insignificant." The group includes some with systolic pressure up to 200 mm. Hg, but diastolic pressures were in most cases less than 120 mm. Hg.

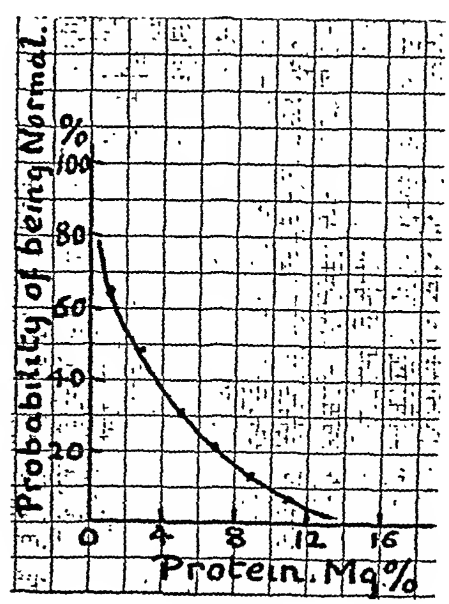

FIG. 5

Results on this group are shown in Figure 6 in the form of a histogram. Where the protein concentration falls outside the normal range (as judged from Figure 4), the blocks are shaded. In 5 cases where the protein concentration was greater than 80 mgm. per cent, these would presumably be noted by the usual clinical tests as having a "trace" of albuminuria. In the 45 other cases, Figure 5 shows that in 15, or 33 per cent, the protein concentration is significantly greater than the normal.

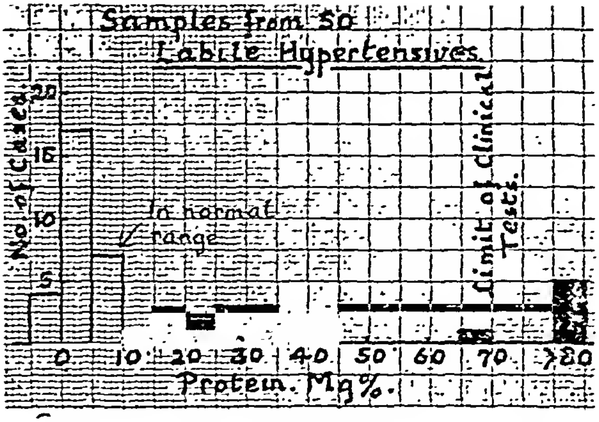

Fig. 6

DISCUSSION

The curve Figure 3 shows that the area of the protein film is not strictly proportional to the amount of protein present, but there is a slight curvature of the graph. This may be attributed to a "concentration effect," which means that, when protein is more concentrated in the liquid in the burette, the ability to form a film is slightly decreased. This is easily understood, since the more competition there is for the surface of the drops as they are formed, the less completely will the protein be adsorbed on the surface of the drops.

The method of measurement of protein concentration by means of the surface activity is widely adaptable to biological fluids other than urine, such as cerebrospinal fluid, blood serum, or lymph. The initial dilution of these fluids should be such that the concentration of proteins in the fluid dropped in the tray is in the same range as in these measurements, *i.e.* not greater than 10 mgm. per cent. This would require an initial dilution of cerebrospinal fluid some 50 times and of blood serum some 200 times. Since different proteins may differ markedly in their surface activity, calibration should be made with standard solutions of the particular protein concerned.

The specificity of the method in measuring protein, and not other substances, is of course the same as the specificity of the precipitation by Tsuchiya's reagent.

From the results on normal urine, a statistical prediction curve can be constructed as in Figure 5 This shows that if a sample of urine is found to contain more than 12 mgm. per cent of protein, the chance that this sample is from a "normal" is less than 5 per cent, and for greater amounts, the chance is insignificant. This gives a statistically valid basis for the use of the term "insignificant" in connection with albuminuria, whereas, on the basis of the usual clinical tests, "insignificant" really referred to the appearance of the precipitate in the particular test used and not to the degree of albuminuria.

The amount of protein in normal urine is so small that it would be extremely difficult to establish that what little was present was not extraglomerular in origin, rather than having passed through the glomerular filter. The view that the normal glomerular membrane is "completely" impermeable to protein is not contradicted by these experiments.

The results on the small group of hypertensives are included merely to indicate that a more sensitive method of measurement of urinary protein is of clinical usefulness. The sensitivity of a clinical test should be at least sufficient to give a definite indication with the amount of substance found in normal physiology so that the degree of deviation from the normal can be judged.

SUMMARY AND CONCLUSIONS

1. Since protein is highly active in forming monomolecular surface films, its concentration in urine can be found by a measurement of the total surface area occupied at a definite surface force, compared with the surface area occupied after protein has been precipitated. For clinical use, it is seldom necessary to take this second step of the precipitation of protein.

2. A simple method, using relatively inexpensive

apparatus, is described. Less than 1 ml. of a sample of urine is required, and the measurement is completed in less than 20 minutes.

3. The method has been used to find the concentration of protein in samples of 100 normal urines. The most frequently found concentration is less than 2 mgm. per cent, the mean is 3.7 mgm. per cent, and the probability of finding more than 12 mgm. per cent in normal samples is less than 5 per cent.

4. When the method is applied to a group of 50 hypertensives, most of them of the labile type, where there was no evidence of albuminuria by the usual clinical tests, amounts of protein significantly greater than normal were found in 33 per cent of these cases.

5. It is concluded that the usual clinical tests for proteinuria applicable on small samples of urine, have a sensitivity which is inadequate to their use. The method employing the surface activity of protein is rapid, easy to perform, requires a very small sample, and is accurate to 2 or 3 mgm. per cent. It can be adapted readily to the measurement of the concentration of protein in other biological fluids.

ACKNOWLEDGMENT

A major part of the technical work was done by Mr. Milton Walsh, and he also contributed a great deal in the development of the method. Thanks are also due to the Medical Staff of the Victoria Hospital and of the Westminster Hospital, London, for their cooperation.

BIBLIOGRAPHY

1. Walker, A. M., Bott, P. A., Oliver, J., and MacDowell, M. C., The collection and analysis of fluid from single nephrons of the mammalian kidney. Am. J. Physiol., 1941, 134, 580.
2. Bott, P. A., and Richards, A. N., The passage of protein molecules through glomerular membranes. J. Biol. Chem., 1941, 141, 291.
3. Meakins, J. C., The Practice of Medicine. C. V. Mosby Co., St. Louis, 1944, 4th Edition.
4. Levinson, S. A., and MacFate, R. P., Clinical Laboratory Diagnosis. Lea & Febiger, Philadelphia, 1943, 2nd Edition, p. 385.

5a. Everett, M. R., Medical Biochemistry. Paul B. Hoeber Inc., New York, 1946, 2nd Edition, p. 445.

5b. Kolmer, J. A., Clinical Diagnosis by Laboratory Examinations. D. Appleton-Century Co., New York, 1943, p. 1006.

6a. Starr, I. Jr., The production of albuminuria by renal vasoconstriction in animals and in man. J. Exp. Med., 1926, 43, 31.

6b. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Williams & Wilkins Co., Baltimore, 1931, Vol. 1, p. 702.

7. Bodansky, M., Introduction to Physiological Chemistry, Wiley and Sons Inc. New York, 1938, 4th Edition.
8. Kolmer, J. A., and Boerner, F., Approved Laboratory Technique. D. Appleton-Century Co., New York, 1945, 4th Edition, p. 119.
9. Mörner, K. A. J., Untersuchen über die Proteïnstuffe und die eiweissfällenden Substanzen der normalen Menschenharns. Skand. Arch. f. Physiol., 1895, 6, 332.
10. Bazett, H. C., and Burton, A. C., Report on the effect of nitrogen liberation on cerebrospinal fluid. Minutes of 4th Meeting of the Associate Committee on Aviation Medical Research, National Research Council of Canada, Appendix T., June 6, 1941.
11. Gorter, E., and Grendel, F., Die Eiweissausbreitung als Methode zur Bestimmung von Serumalbumin und Serumglobulin. Biochem. Ztschr., 1928, 201, 301.
12. Pockels, A., Nature, 1891, 43, 437.
13. Adam, N. K., The Physics and Chemistry of Surfaces. Oxford University Press, London, 1941, 3rd Edition, p. 87.
14. Blodgett, K. B., Interference colors in oil films on water. J. Optic. Soc. America, 1934, 24, 313.
15. Langmuir, T., and Schaeffer, V. J., Properties and structure of protein monolayers. Chem. Rev., 1939, 24, 181.
16. Folin, O., and Denis, W., The quantitative determination of albumin in urine. J. Biol. Chem., 1914, 18, 273.

THE EFFECT OF CYTOCHROME C UPON THE METABOLISM OF RAT TISSUES

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Recently, Proger and his co-workers have published papers dealing with the effects of parenteral injections of cytochrome C on conditions associated with tissue anoxia (1 to 8). They found, in man, that the changes in the visual acuity, electrocardiogram, and electroencephalogram associated with anoxia were partially or completely prevented by the antecedent intravenous injection of relatively small amounts of cytochrome C.

These phenomena were explained by Proger by assuming that in anoxia essential oxidative processes were limited by the low concentration in the cells of cytochrome C *relative* to cytochrome oxidase. By parenteral injection the concentration of cytochrome C within the cells was increased, the limitation of oxidative processes was removed, and tissue function restored toward normal.

Proger discussed further experiments bearing on the problem of the role of cytochrome C in tissue anoxia: (*1*) He reported (5) that the adenosine triphosphate (ATP) of heart and kidney of rats subjected to anoxia was markedly reduced. The antecedent parenteral injection of cytochrome C significantly prevented this reduction. It is well known that the organic phosphates, particularly ATP, play important roles in tissue metabolism. The regeneration of these energy rich phosphate bonds is dependent upon oxidative processes. Presumably, then, increase of cellular cytochrome C by injection removed the limiting factor of its naturally occurring low concentration, increased oxidative processes associated with the regeneration of ATP, and thus prevented the decrease otherwise accompanying anoxia. (*2*) He also found that cytochrome C together with hydroquinone (4) increased the oxygen uptake of *homogenized* tissues *in vitro*.

These 2 experiments were considered by Proger to support his concept of the role of parenterally administered cytochrome C in combating tissue anoxia. There are, however, theoretical difficulties in the acceptance of this explanation, viz.: (*1*) How can the large molecule of cytochrome C (molecular weight approximately 13,000) enter into the interior of the cell in order to function? (*2*) Is the cytochrome C concentration of tissues so low that it is a limiting factor in metabolism under normal extraordinary physiological demands?

In this paper the interesting problem of the possible beneficial effect of parenteral injections of cytochrome C in anoxic states was studied in 3 ways: (*1*) The adenosine triphosphate (ATP) concentration of kidney tissue in rats in the normal state, in anoxia, and in anoxia subsequent to cytochrome C administration was studied. (*2*) Experiments were performed to determine the effect of cytochrome C on the oxygen uptake *in vitro* of diaphragms, liver and kidney slices from rats. (*3*) Using a recent value for the turnover number of cytochrome C together with cytochrome C concentrations of various tissues, the limiting values of oxygen uptake imposed by these 2 factors was calculated and compared with the physiological range of oxygen uptake of the respective tissues.

METHODS

The concentration of inorganic phosphate, ATP, and total phosphate in the acid-soluble fraction of the kidney was determined by the methods of Kaplan and Greenberg (9). One hundred-minute hydrolyzable phosphate was determined by hydrolysis in 1N H_2SO_4 at 100° C. All phosphate determinations were by the method of Fiske and Subbarow (10), using a Klett-Summerson photoelectric photometer with a 6,600 A° filter.

The rats were made anoxic for 5 minutes by means of a 3 per cent O_2 − 97 per cent N_2 gas mixture passed through a funnel held over the animal's head. Cytochrome C was injected intravenously 10 minutes prior to the anoxic period in the manner reported by Proger *et al* (5).

The kidney was frozen with liquid air as quickly as possible following decapitation of the rat. The frozen kidney was ground to a powder under liquid air in a mortar, and this powder was added to a tared centrifuge

tube containing 7 per cent trichloroacetic acid, following the procedure of Kaplan and Greenberg (9).

The cytochrome C was prepared according to the technique of Keilin and Hartree (11). The concentration was determined spectrophotometrically, using the constant 26.1 at λ 550 mμ for a millimolar solution of cytochrome C, with a cuvette depth of 1 cm. This concentration is referred to pure cytochrome C with 0.43 per cent Fe (Mol. wt. = 13,000). In addition the biological activity of the preparation was demonstrated by its ability to increase the oxygen uptake of rat liver homogenates in the presence of 0.01M succinate. The increment was of the order of 1 mole of O_2 per mole of cytochrome C per minute, which is comparable to that obtained by Schneider and Potter (13) with their preparations of cytochrome C.

In the experiments dealing with tissue slice respiration, the organs were removed immediately following decapitation and slices 0.35 mm. in thickness were cut. The slices were placed in Warburg vessels containing 2 ml. of a medium of the following composition: Na_2HPO_4, 0.020M; NaH_2PO_4, 0.020M; KCl, 0.005M; $MgCl_2$, 0.002M; $CaCl_2$, 0.001M; NaCl, 0.09M; glucose, 0.011M; pH = 6.7. Where noted, the glucose was replaced by succinate.

RESULTS

The effect of cytochrome C on the ATP content of kidneys from normal and anoxic rats

The results presented in Table I show that the anoxia caused a 74 per cent decrease in the ATP content of the kidneys of the normal, non-injected rats. The 100-minute minus the 7-minute easily hydrolyzable phosphate, representing mainly hexose diphosphate, was decreased 52 per cent. There was no significant change in the inorganic and total phosphate content.

The rats made anoxic following injection of cytochrome C showed a similar reduction in the ATP and hexose diphosphate content of the kidney. There is no statistically significant difference in the data of the untreated and treated rats.

TABLE I

Partition of the organic acid-soluble phosphates (mgm. P per 100 grams wet weight) of kidneys from normal rats, anoxic rats, and rats previously injected with 5 mgm. of cytochrome C

Condition of rat	Inorganic phosphate	7-minute hydrolyzable phosphate (ATP)	100-minute minus 7-minute hydrolyzable phosphate	Total phosphate
	mgm. P	*mgm. P*	*mgm. P*	*mgm. P*
Normal	41.9	12.4		89.4
	31.6	5.6	10.8	90.7
	32.9	11.3	11.3	114.0
	32.0	13.0	7.3	93.1
	24.0	6.2	3.7	78.0
	28.8	6.4	8.0	93.0
Anoxic	27.8	8.0	3.3	99.5
	31.8	0.9	8.6	92.0
	33.2	0.0	3.4	82.0
	37.6	0.9	3.8	129.0
	33.1	0.9	1.2	112.0
	24.3	2.3	3.9	95.0
	13.8	3.5	3.1	123.0
Anoxic, previously injected with 5 mgm. of cytochrome C	37.8	2.6	7.8	85.1
	37.9	2.4	4.9	91.5
	44.6	0.0	3.9	90.0
	28.2	3.4	5.6	118.0
	23.1	1.4	4.2	94.5
	23.6	4.7	4.5	112.0
	24.4	2.6	2.2	106.5
Normal, mean ± S.E.	31.9 ± 2.5	9.2 ± 1.4	8.2 ± 1.4	93.0 ± 4.8
Anoxic, mean ± S.E.	28.8 ± 2.9	2.4 ± 1.0	3.9 ± 0.6	104.6 ± 6.5
Anoxic, previously injected with cytochrome C, mean ± S.E.	31.4 ± 3.2	2.4 ± 0.6	4.7 ± 0.5	99.7 ± 4.7

The effect of cytochrome C upon oxygen uptake of tissues in vitro

It is well known (*e.g.*, Elliott [12]) that the addition of cytochrome C (together with some reductant such as hydroquinone or ascorbic acid) to *homogenized* tissue *in vitro* increases the oxygen uptake. The currently accepted explanation is that the cytochrome oxidase is in excess compared to cytochrome C. Hence in the homogenate, in which both cytochrome oxidase and cytochrome C are *free*, the further addition of cytochrome C saturates the cytochrome oxidase to a greater extent and oxygen uptake is enhanced. Proger (4) repeated experiments of this kind with homogenates and used them to support his general concept of the effect of cytochrome C on anoxia in the intact animal. It seems to the authors of this paper that such a phenomenon in homogenates has little or no meaning with respect to the problem at hand unless it can be shown that parenterally administered cytochrome C enters the intact cell. Elliott (12) was unable to find any influence of cytochrome C upon the O_2 uptake *in vitro* of intact tissue and concluded that cytochrome C does not penetrate into intact cells.

To test whether the addition of cytochrome C to the medium would have any effect upon the oxygen uptake, we used relatively untraumatized tissue preparations such as kidney or liver slices, or whole rat diaphragms rather than homogenates. We found, in the presence of glucose or succinate, that cytochrome C has no significant effect. The evidence is presented in Table II.

TABLE II

The effect of cytochrome C upon the oxygen uptake in vitro *of diaphragms, kidney and liver slices from rats*

Expt. No.	Tissue preparation	Medium employed	Oxygen uptake, no cytochrome C	Change in oxygen uptake in presence of cytochrome C
			μM O_2 per gram wet weight per hour	*per cent*
1	Diaphragm	Medium I*	85.7	11
2			100.0	−14
3			75.4	8
4			70.6	15
5			71.4	5
6			77.8	−5
7			82.4	−3
8			59.4	0
9			58.6	−2
			Mean	+1.7
10	Kidney slices		158.5	−13
11			147.6	−7
			Mean	−10.0
12	Liver slices		65.6	6
13			69.1	−2
14			87.8	−16
			Mean	−4.0
15	Diaphragm	Medium II*	58.3	8
16			79.3	−10
17			79.5	−13
18			68.1	0
19			66.0	11
			Mean	−4.0

* Medium I contained *glucose*, 0.011M; Na_2HPO_4, 0.02M; NaH_2PO_4, 0.02M; KCl, 0.005M; $MgCl_2$, 0.002M; $CaCl_2$, 0.001M; NaCl, 0.09M. pH = 6.7. Medium II contained sodium *succinate*, 0.01M in place of the glucose in Medium I. Total vol. 2.5 ml. Cytochrome C 1.7 mgm. t = 38° C.

The limiting cellular oxygen uptake imposed by cytochrome C content

Proger (8) emphasized that the concentration of cytochrome C in tissues is below that required for maximum activation of cytochrome oxidase with which it is coupled in metabolic reactions. This, however, does not indicate that cytochrome C is necessarily a limiting factor in cellular oxygen consumption. From the data of Rosenthal and Drabkin (14) the turnover number of cytochrome C expressed in terms of oxygen uptake is 2.3×10^7 μM of O_2 per gram (dry weight) per hour. On the basis of this figure we have calculated the values shown in Table III. These calculations indicate that the cytochrome C concentration of various rat organs is considerably beyond that required for the estimated physiological demand.

TABLE III

The calculated maximum oxygen uptake of various tissues calculated from the turnover number and tissue concentrations of cytochrome C compared to physiological ranges of oxygen uptake

Organ	Cytochrome C	Calculated oxygen uptake	Approximate physiological range of oxygen uptake
	*per cent of dry weight**	Q_{O_2}	Q_{O_2}
Kidney cortex	0.14	720	10–50
Liver	0.06	309	10–30
Brain cortex	0.04	206	10–40
Striated muscle	0.05	257	5–110
Heart	0.22	1133	3–60

* From data from rats by Rosenthal and Drabkin (15). Expressed in terms of oxygen uptake the turnover number of cytochrome C used was 2.3×10^7 μM of O_2 per gram dry weight per hour. The calculation of the Q_{O_2} is: $2.3 \times 10^7 \times 22.4 \times 10^{-3} \times P \times 10^{-2}$ where P is the per cent (dry weight) of cytochrome C in the respective tissue. Q_{O_2} = cu. mm. O_2 per mgm. dry weight per hour.

In the brain, for example, there appears to be a 5-fold reserve factor, and in striated muscle, the factor is 2 even under the demands of excessive muscular work. In the heart the factor of safety appears to be still greater, viz. 20. It is difficult, therefore, to understand, on the basis of the current concept of the function of cytochrome C in tissue oxidations, how the parenteral addition of a relatively small amount of cytochrome C, even if it could enter the cell, can affect oxidative cellular metabolism.

SUMMARY

1. The decreased adenosine triphosphate (ATP) content of anoxic rat kidney reported by Proger *et al* (5) has been confirmed. The prevention of the lowered ATP content of anoxic kidney by the antecedent intravenous administration of cytochrome C, reported by these authors, has not been observed in the present experiments.

2. No alteration in the oxygen consumption of rat diaphragm and rat liver and kidney slices was observed following the addition of cytochrome C to the medium *in vitro* either with glucose or succinate as substrates.

3. Using the turnover number of cytochrome C and known values of tissue cytochrome C, the maximum oxygen uptake of various tissues was calculated. Comparison of these calculated values with the range of oxygen requirements lends no support to the concept that the cytochrome C con-

tent of tissues is a limiting factor in cellular oxidations within physiological ranges.

We are indebted to Dr. David L. Drabkin of the Department of Physiological Chemistry for the spectrophotometric standardization of the cytochrome C solutions.

BIBLIOGRAPHY

1. Proger, S., Aisner, M., and Squires, R. B., The prevention of electrocardiographic evidences of myocardial anoxemia by chemical means. J. Clin. Invest., 1942, *21*, 630.
2. Proger, S., The use of respiratory catalysts, particularly succinic acid in tissue anoxia. Bull. New England M. Center, 1943, *5*, 80.
3. Proger, S., Dekaneas, D., and Schmidt, G., The increase of cytochrome C content of organs following its parenteral injection. J. Clin. Invest., 1944, *23*, 949.
4. Proger, S., Dekaneas, D., and Schmidt, G., Some observations on the effect of injected cytochrome C in animals. J. Clin. Invest., 1945, *24*, 864.
5. Proger, S., Dekaneas, D., and Schmidt, G., The effects of anoxia and of injected cytochrome C on the content of easily hydrolyzable phosphorus in rat organs. J. Biol. Chem., 1945, *160*, 233.
6. Proger, S., and Dekaneas, D., The clinical use of cytochrome C in patients with intermittent claudication. Bull. New England M. Center, 1946, *8*, 145.
7. Proger, S., and Dekaneas, D., Some further observations on the parenteral use of cytochrome C with special reference to cerebral anoxia and shock. Bull. New England M. Center, 1945, *7*, 149.
8. Proger, S., and Dekaneas, D., The use of cytochrome C in combating tissue anoxia. Science, 1946, *104*, 389.
9. Kaplan, N. O., and Greenberg, D. M., Studies with radioactive phosphorus of the changes in the acid-soluble phosphates in the liver coincident to alterations in carbohydrate metabolism. I. Separation and nature of the organic acid-soluble phosphates of liver. J. Biol. Chem., 1944, *156*, 511.
10. Fiske, C. H., and Subbarow, Y., The colorimetric determination of phosphorus. J. Biol. Chem., 1925, *66*, 375.
11. Keilin, D., and Hartree, C. F., Proc. Roy. Soc. London, Series B, 1937, *122*, 298.
12. Elliott, K. A. C., and Greig, M. E., The distribution of the succinic oxidase system in animal tissues. Biochem J., 1938, *32*, 1407.
13. Schneider, W. C., and Potter, V. R., The assay of animal tissues for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem., 1943, *149*, 217.
14. Rosenthal, O., and Drabkin, D. L., Cytochrome C, cytochrome oxidase and body size. Fed. Proc., 1943, *2*, 69.
15. Rosenthal, O., and Drabkin, D. L., The cytochrome C content of normal and neoplastic mammalian epithelium, and its correlation with body mass. J. Biol. Chem., 1943, *150*, 131.

THE RENAL CLEARANCE OF PENICILLINS F, G, K, AND X IN RABBITS AND MAN[1]

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The factor which most seriously limits the therapeutic efficacy of penicillin is its rapid urinary excretion. Thus, the renal clearance of commercial penicillin in man has been found (1, 24) to vary between 755 and 1,120 ml. per minute, approximating the total renal plasma flow; and when crystalline penicillin G or X is injected intramuscularly in aqueous solution at average therapeutic dosage (0.6 mgm. per kgm.), approximately 60 per cent is excreted in the urine in the first hour (2 to 4). Largely because of that rapid excretion, the blood level of *e.g.* penicillin G falls off, after its intramuscular injection in aqueous solution, at an average rate of 70 to 80 per cent each succeeding hour, or 2 to 3 per cent each minute (4, 19). It is apparent that even a minor decrease in the rate of renal excretion would be reflected in significantly prolonged blood levels and a correspondingly enhanced therapeutic efficacy.

There is considerable evidence that penicillin is secreted by the renal tubules, and by the same secretory mechanism as diodrast and p-aminohippuric acid. Thus, the excretion of penicillin is said to be depressed by the simultaneous administration of either of these 2 compounds in amounts sufficient to saturate the common tubular mechanism (5 to 10). Restriction of the water or salt intake (11) and the administration of benzoic acid or pitressin (12) are also reported to curtail the excretion of penicillin and to prolong the blood penicillin curve correspondingly.

Since the 4 penicillin species known to be produced by penicillium (F, G, K, and X) vary markedly in their pharmacological behavior and bactericidal activity (2 to 4, 13, 14), it was of interest to determine the renal clearance of each, over a widely varying range of plasma concentration, and with varying rates of urine flow. As is shown in the following, in both rabbits and man penicillins F, G, and X were found to have a renal clearance corresponding to the total renal plasma flow. The ratio of the glomerular filtration rate, simultaneously determined with inulin or thiosulfate, to the renal clearance of penicillin did not vary significantly over a wide range of plasma concentrations and was independent of the rate of urine flow. In rabbits, the tubular secretory mechanism was found to have been saturated by serum concentrations on the order of 100 to 1,000 micrograms per ml.

The apparently anomalous renal clearance of penicillin K is discussed later in the text.

At least in the case of penicillins F, G, and X the nature of the side chain which differentiates the several species of penicillin, and which significantly modifies their bactericidal activity, thus has no demonstrable effect on their renal clearance. Further modification in that prosthetic group may nevertheless be a promising approach to the development of penicillins with low renal clearances and correspondingly enhanced therapeutic activity.

EXPERIMENTAL METHODS

Penicillins: The purified penicillins used in these studies were generously provided by the following pharmaceutical firms:

G—Squibb (Lot CRA-214-20)
F—Upjohn (Lot 175-EANW-6)
K—Abbott (Lot RP 309 P2)
Pfizer (Lot 5/2/46)
X—Lederle (Lot CA-3242-IC)

Penicillin assay: The blood and urine concentrations were determined by a modified Rantz-Kirby (1) serial dilution technic (cf. [4, 13]), using the inhibition of hemolysis by streptococci (C-203 strain of Streptococcus pyogenes) as the endpoint. The use of 5 tubes for each

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2-fold difference in concentration (*i.e.*, 0.8, 0.72, 0.6, 0.48, 0.4, etc. ml. of the unknown specimen in a total volume of 0.8 ml.) permitted a reasonably accurate and reproducible assay.

The technic was further modified in that the assays were removed from the incubator after 6 to 9 hours, by which time hemolysis had begun, and were then allowed to remain at room temperature overnight before reading. With this technic, contaminated urine specimens could usually be assayed without preliminary filtration, since the heavy streptococcus inoculum initiated hemolysis at 37° C. before it had been overgrown by the contaminant, and bacterial growth in the following 10 to 15 hours at room temperature was usually too slow to obscure the results.

Calculation of results: The average serum concentration over the time period represented by the successive urine samples was obtained in the usual manner by graphic interpolation (log of serum level plotted against time).

Calibration of glomerular filtration and renal plasma flow: In some of the experiments, the rate of glomerular filtration was simultaneously determined by the administration of inulin or sodium thiosulfate, given either as a single injection, or as a constant infusion following a priming dose. In some of the subjects, the renal plasma flow was determined with para-aminohippuric acid several days before or after the administration of penicillin. The methods used for the determination of inulin and para-aminohippuric acid were those suggested by Goldring and Chasis (16). Thiosulfate was determined by the method of Newman (17).

EXPERIMENTAL

Group I. Human subjects continuously infused with penicilllin F, G, K, or X

The results in 6 experiments are given in detail in Table I and are graphically summarized in Figures 1 to 3.

In subject G. J., the renal clearance of penicillin G averaged 525 ml. per minute. This is to be compared with a calculated renal plasma flow of 717 ml. per minute and an observed clearance for para-aminohippuric acid of 619 ml. The parallelism between the renal clearance of penicillin G and the glomerular filtration rate simultaneously determined with inulin (*cf.* last 2 columns of Table I and Figure 1), and the constancy of their ratio (0.19 to 0.24, averaging 0.23) are particularly to be noted.

The renal clearance of penicillin G in this subject was independent of the blood level and the rate of urine flow, within the experimental range. Periods in which the blood levels averaged 2.4 and 0.25 micrograms per ml. gave clearances of 498 and 416 ml. per minute, respectively; and periods in which the urine flow was 12.5 and 2.1 ml. per minute gave penicillin clearances of 588 and 416 ml. per minute, respectively.

When the same subject was infused with penicillin X (bottom of Table I), the renal clearance averaged 652 ml. per minute, in satisfactory agreement with both the calculated renal plasma flow (717) and the observed clearance of para-aminohippuric acid (619), and 4.6 times the calculated glomerular filtration rate of 135 ml. per minute. The renal clearance was again largely independent of the absolute serum concentration and rate of urine flow. With a 10-fold difference in serum concentration (3.6 and 0.37 microgram per ml.), the renal clearance varied from 456 to 699 ml. per minute; and in periods in which the urine flow averaged 10.3 and 4 ml. per minute, the renal clearances averaged 787 and 722 ml., respectively.

Subject E. W. is of particular interest (Figure 1 and the second section of Table I). This subject was a woman with long-standing hypertension, in whom the urea clearance was 58 per cent of normal, and the phenolsulfaphthalein excretion was 60 per cent in 2 hours. Corresponding to the obvious impairment of renal function, the renal clearance of penicillin G averaged 242 instead of the calculated value of 617, or 40 per cent of normal. Significant also is the fact that as her blood pressure fell from 218/112 to 158/114 during the first 80 minutes of the experiment, the inulin clearance increased from 52 to 97, and the penicillin clearance from 158 to 308, indicative of an increased flow of blood to the kidney. Meanwhile, the ratio of glomerular filtration to total renal clearance remained essentially constant at the abnormally high level of 0.31. As in patient G. J., the rate of urine flow had no significant effect on renal clearance, which varied only from 276 to 230 ml. per minute as the urine flow increased from 2.0 to 7.4 ml. per minute in 5 successive experimental periods.

Penicillin F gave results essentially the same as penicillins G and X. The renal clearances averaged 550 and 900 ml. per minute in 2 patients in whom the calculated renal plasma flow was 750 ml. per minute. The clearance was again independent of the absolute blood level and of the rate of urine flow over the entire experimental range (*cf.* Figure 3 and Table I).

In patient M. B., injected with penicillin F, midway through the experiment the blood flow to the kidney apparently decreased sharply for a period of approximately 40 minutes. This was manifested by a simultaneous and parallel decrease in the renal clearance of *both* penicillin

TABLE I

The renal clearance of penicillins F, G, K and X in man

Experiments with continuous intravenous infusion at a falling rate. Most of the patients received a priming dose of 2.5 mgm. per kgm. and the rate of infusion was slowly reduced from an initial level of 1 mgm. per kgm. per min. to 0.16 mgm. per kgm. per min. over a period of 2 to 3 hours. In all but 2 patients, the urine specimens were collected by catheterization.

Penicillin species	Subject	Urine collection: Time period	Urine collection: Urine flow	Urine penicillin	Average serum penicillin	Renal clearance of penicillin	Glomerular filtration rate, experimentally detd. with inulin or thiosulfate	Ratio of glomerular filtration to penicillin clearance
		minutes	*ml. per min.*	*µg. per min.*	*µg. per ml.*	*ml. per min.*	*ml. per min.*	
F**	H. G.	30–50	9.6	550	0.77	714		0.20
	Wt. = 75 kgm.	50–74	10.2	221	0.53	417	142	0.34
	Ht. = 70 in.	74–97	6.1	222	0.41	541	(calcd. from	0.26
	Surface area =	97–114	9.7	156	0.29	540	surface area)	0.26
	1.89 sq. m.	114–130	11.5	111	0.26	426		0.33
		130–142	15.7	165	0.25	660		0.22
					Experimental averages	550		0.26
					Calculated from surface area	750	142	
	M. B.	19–38	0.8	540	0.6	900	121	0.14
	Wt. = 75.5 kgm.	38–56	1.4	300	0.32	937	186	0.20
	Ht. = 68 in.	56–79	6.7	227	0.21	1080	216	0.20
	Surface area =	79–98	4.4	55	0.15	365	116	0.32
	1.89 sq. m.	98–118	0.9	32	0.11	291	100	0.34
		118–137	2.9	55	0.074	737	137	0.18
		137–157	7.5	44	0.052	850		
					Experimental averages	900*	165*	0.18
					Calculated from surface area	750	142	
G	G. J.	24–44	7.9	1195	2.4	498	104	0.21
	Wt. = 65 kgm.	44–63	9.1	790	1.4	564	123	0.22
	Ht. = 69½ in.	63–84	12.5	646	1.1	588	131	0.22
	Surface area =	84–104	8.5	437	0.82	533	118	0.22
	1.78 sq. m.	104–125	3.4	381	0.56	670	130	0.19
		125–144	4.6	274	0.48	571	134	0.24
		144–163	2.3	181	0.41	441		
		163–183	2.4	146	0.335	436		
		183–203	2.1	104	0.25	416		
					Experimental averages	525	123	0.23
					Calculated from surface area	717	135	0.19
					PAHA clearance	619		
	E. W.	34–55†	3.2	1438	9.1	158	52	0.33
	Wt. = 78 kgm.	55–74	2.0	1516	5.5	276	76	0.27
	Ht. = 63 in.	74–95‡	3.4	1200	3.9	308	97	0.31
	Surface area =	95–114	4.9	787	2.9	271		
	1.8 sq. m.	114–134	6.7	556	2.25	247		
		134–155	7.4	387	1.68	230		
		155–174	5.2	281	1.2	234		
		174–194	3.0	198	0.93	213		
	Urea clearance = 50 per cent of normal				Experimental averages	242	75	0.31
	Phenolsulfonphthalein = 60 per cent in 2 hrs.				Calculated from surface area	617	126	0.20

TABLE I—*Continued*

Penicillin species	Subject	Urine collection: Time period	Urine collection: Urine flow	Urine penicillin	Average serum penicillin	Renal clearance of penicillin	Glomerular filtration rate, experimentally detd. with inulin or thiosulfate	Ratio of glomerular filtration to penicillin clearance
		minutes	*ml. per min.*	*µg. per min.*	*µg. per ml.*	*ml. per min.*	*ml. per min.*	
K		35–53	9.5	983	2.6	378		
		53–70	2.7	433	2.1	206		
	E. P.	70–96	1.4	176	1.6	110		
	Wt. = 94 kgm.	96–112	6.0	418	1.1	380	160	
	Ht. = 70 in.	112–140	4.4	309	0.89	348	(calcd. from	0.60
	Surface area =	140–161	2.3	152	0.63	241	surface area)	
	2.11 sq. m.	161–180	0 8	64	0.41	151		
		180–200	1.1	63	0.27	233		
		200–219	?	45	0.11	406		
					Experimental averages	272		0.60
					Calculated from surface area	850	160	
		25–67	2.4	481	2.55	189	74	0.39
		67–86	2.3	483	2.0	241	71	0.29
	W. J.	86–108	5.7	510	1.65	309	112	0.36
	Wt. = 57 kgm.	108–129	5.0	414	1.22	340	115	0.34
	Ht. = 66 in.	129–153	4.0	326	0.90	362	111	0.31
	Surface area =	153–178	4.4	179	0.69	260	108	0.41
	1.62 sq. m.	178–197	7.8	172	0.56	307	143	0.47
		197–224	4.6	116	0.41	283	108	0.38
		224–243	2.6	37	0.29	128	68	0.53
					Experimental averages	269	101	0.39
					Calculated from surface area	665	125	0.20
X		24–41	9.8	1641	3.6	456		
		41–61	4.0	1444	2.0	722		
	G. J.	61–82	5.0	1070	1.6	670		
	Wt. = 65 kgm.	82–101	10.3	1062	1.35	787	135	
	Ht. = 69½ in.	101–117	8.5 } 6.7	1045 } 602	1.15 } 0.96	900 } 627	(calcd. from	0.19
	Surface area =	117–140	4.8 }	307 }	0.84 }	365 }	surface area)	
	1.78 sq. m.	140–162	4.3 } 4.7	208 } 298	0.55 } 0.51	380 } 584		
		162–179	5.4 }	415 }	0.47 }	883 }		
		179–201	5.6	258	0.37	699		
					Experimental averages	652		0.22
					Calculated from surface area	717	135	0.20
					PAHA clearance (detd.)	619		

* Two periods of obviously decreased renal plasma flow were not included in calculating averages.
** Because of the small amounts of penicillin F available, the dosage in these 2 patients was approximately half that used in the experiments with G, K, and X
† Blood pressure 218/112.
‡ Blood pressure 158/114.

and thiosulfate and a significant increase in the ratio of glomerular filtration to total renal clearance. All 3 values returned to normal levels in the last 2 experimental time periods.

Contrasting with the values obtained for the clearance of penicillin G and X, the renal clearance of penicillin K in 2 normal subjects averaged 272

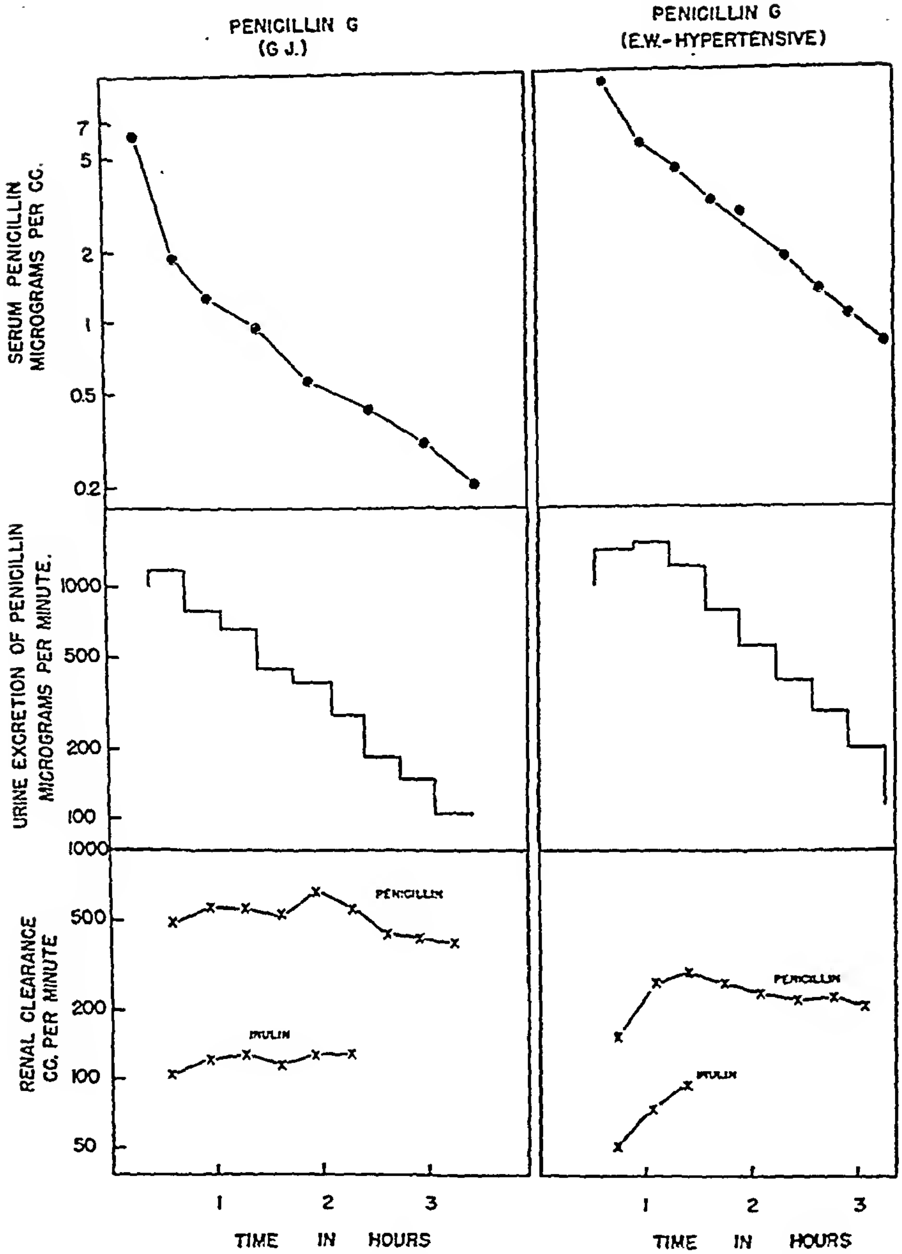

FIG. 1. THE BLOOD LEVEL, URINE EXCRETION AND RENAL CLEARANCE OF PENICILLIN G IN SUBJECTS G. J. AND E. W.
Continuous intravenous infusion at a falling rate (After data of Table I).

and 269 ml. per minute. Within each experiment, however, the results in individual time periods varied widely, the renal clearances varying from 110 to 406 in one subject, and from 128 to 362 in the other. These variations had no demonstrable relationship to either the blood level or rate of urine flow. In these 2 subjects, the calculated renal plasma flow was 850 and 665 ml. per minute, 3 and 2.5 times greater than the clearance of penicillin K. The low values for the renal clearance of penicillin K, which were confirmed in the larger group of patients discussed in the following section, are significant in relation to the low urine recovery of that penicillin species as compared with that of penicillins F, G, or X (*cf.* page 914).

Group II. The renal clearance of penicillins G, K and X in human subjects receiving a single intravenous or intramuscular injection of an aqueous solution

The determination of the renal clearance of penicillin in subjects receiving a single injection of the aqueous solution was far less accurate than the continuous infusion technic used in the experiments of the previous section. The rapidly falling blood level made the "average" value over a given time period of dubious quantitative significance, while a relatively small amount of residual urine in patients who voided voluntarily, or incomplete bladder washing in patients who were catheterized, introduced a large error in the following sample. Despite these limitations, it is clear from the data of Table II that the renal clearance of penicillins G and X generally approximated the total renal

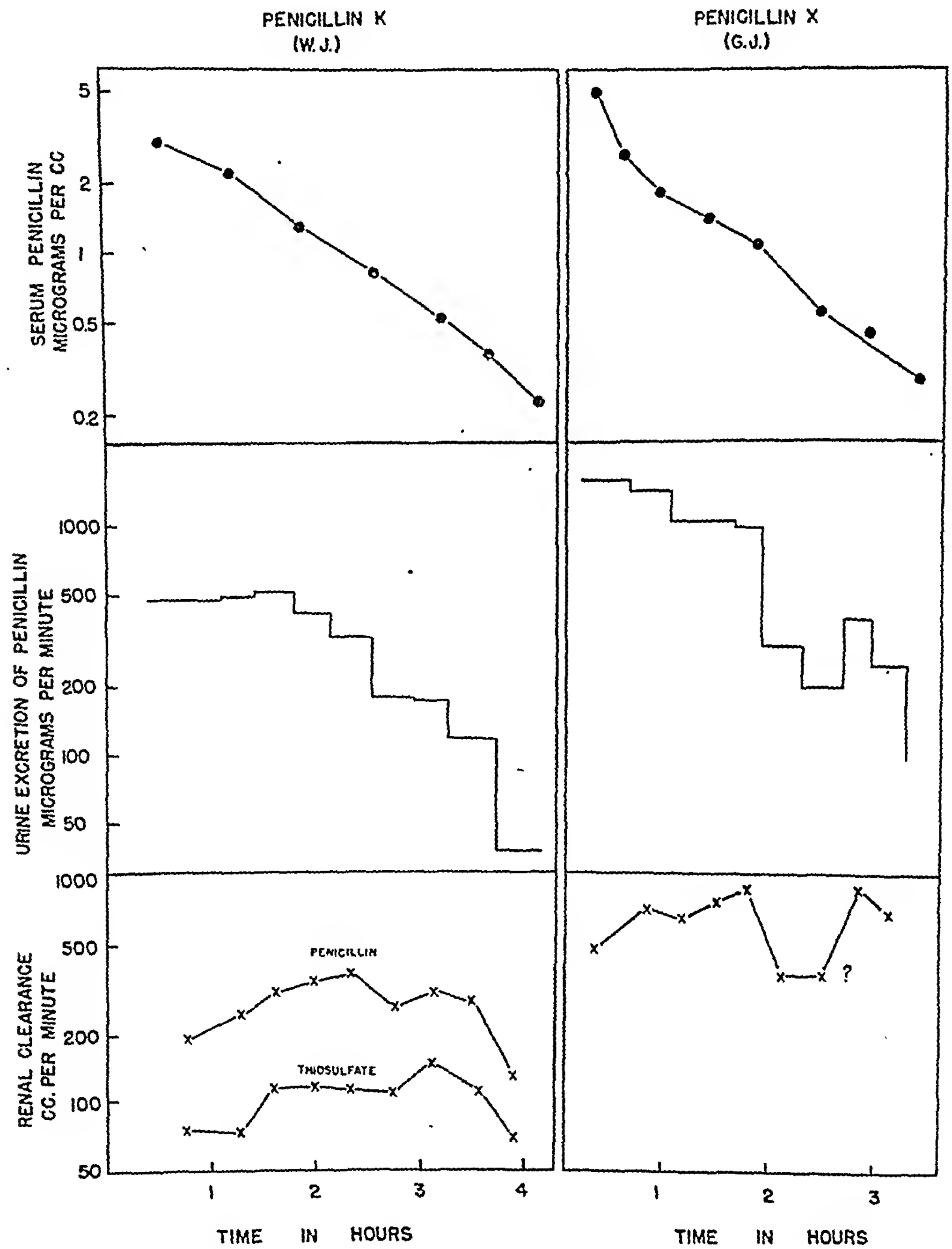

FIG. 2. THE BLOOD LEVEL, URINE EXCRETION AND RENAL CLEARANCE OF PENICILLINS K AND X IN SUBJECTS W. J. AND G. J.

Continuous intravenous infusion at a falling rate (After data of Table I).

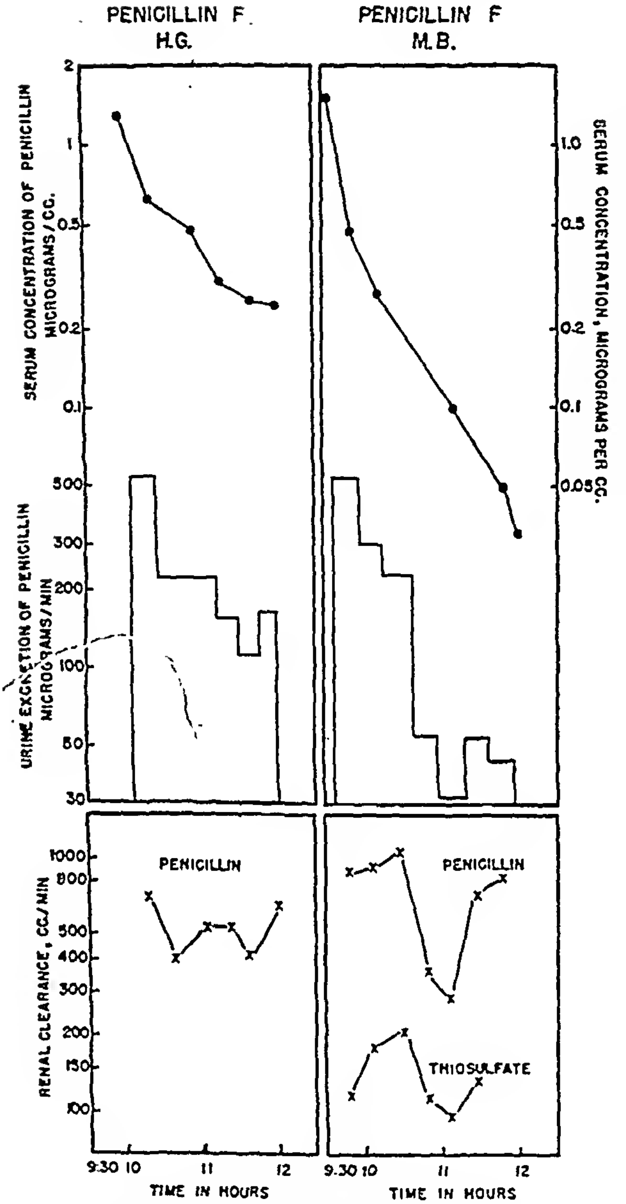

FIG. 3. THE BLOOD LEVEL, URINE EXCRETION AND RENAL CLEARANCE OF PENICILLIN F IN SUBJECTS H. G. AND M. B.

Continuous intravenous infusion at a falling rate (After data of Table I).

plasma flow and averaged 4 to 6 times the glomerular filtration rate.

Patient W. B., an apparent exception, was a hypertensive with a blood pressure of 210/135. The penicillin clearance of G in this patient was considerably less than the calculated normal renal blood flow, and the results in successive time periods were highly variable. However, the ratio of the glomerular filtration rate, experimentally determined with inulin, to the total renal clearance of penicillin was fairly constant, averaging 0.23. The low average clearance in this patient is probably attributable to renal damage associated with long-standing hypertension, and the variability in successive periods can probably be related to the patient's apprehensiveness and incomplete voiding.

Penicillin K again gave results differing from those obtained with penicillins G and X. In the 5 patients studied, the observed renal clearance of K averaged 0.3, 0.2, 0.9, 0.5 and 0.25 of the calculated renal plasma flow. It is particularly to be noted that of these 5 patients, one (D.), when tested with G, gave a normal clearance of 850 ml. per minute, 4 times the value obtained with K; and a second patient (J. W.), who gave an average clearance of 165 ml. per minute with penicillin K, gave clearances of 600 and 1010 ml. per minute when tested with X.

Group III. The renal clearance of penicillin G in in man after its injection as a suspension in peanut oil and beeswax

In 2 patients, the renal clearance of penicillin G was determined after its injection as a finely divided suspension of the potassium salt in peanut oil and 4.8 per cent beeswax (Romansky formula [15]). The prolonged and slowly falling blood levels obtained with this preparation proved admirably suited to the study of renal clearances. In 2 such experiments, summarized in Table III, the average renal clearances were 687 and 471 ml. per minute, to be compared with the calculated renal plasma flows of 796 and 632. These clearances were 4.2 and 3.3 times the glomerular filtration rate as determined with sodium thiosulfate.

Group IV. The renal clearance of penicillin in rabbits

The results obtained in rabbits injected intramuscularly with the crystalline penicillins are summarized in Table IV. The renal clearance of penicillins F, G, and X, studied in a total of 17 rabbits which received 0.35 to 60 mgm. per kgm., varied from 18 to 100. In 4 rabbits receiving penicillin F, the renal clearance averaged 32 ml. per minute (15 to 46 ml. per minute), in 8 rabbits receiving penicillin G at dosages of 0.6 to 60 mgm. per kgm. the average clearance was 59 ml. per minute (17 to 111 ml. per minute), and in 5 rabbits receiving penicillin X the average clearance

was 36 ml. per minute (23 to 54 ml. per minute). These results are to be compared with a reported (18) renal plasma flow in rabbits of 1.5 to 4.8 ml. per minute per gram kidney, or a range of 24 to 77 ml. per minute in rabbits with a total kidney weight of 16 grams.

One can only speculate as to the degree to which the technical manipulations of repeated catheterization and cardiac punctures affected the blood flow to the kidney, and contributed to the discrepant results sometimes obtained in successive time periods in the same animal. In most of the experiments the renal clearance of penicillin probably approximated the renal plasma flow; while the significantly lower values sometimes obtained may reflect a decreased blood flow to the kidney under the conditions of the experiment.

Five rabbits were injected with penicillin K at 0.6 mgm. per kgm. However, the speed with which the blood levels fell and the short periods of

TABLE II

The renal clearance of penicillins G, K and X in man

Single intramuscular or intravenous injection. All urine specimens collected by spontaneous voiding, and not by catheterization.

Penicillin species	Subject	Time period	Urine flow	Urine penicillin	Average serum penicillin	Renal clearance of penicillin: Experimental (Aver.)	Renal clearance of penicillin: Calculated from surface area	Rate of glomerular filtration: Experimentally detd. with inulin	Rate of glomerular filtration: Calculated from surface area	Ratio of glomerular filtration to penicillin clearance
		hours	*ml. per min.*	*µg. per min.*	*µg. per ml.*					
G	W. B. (hypertension) Wt. = 73 kgm. Ht. = 67¾ in. Surface area = 1.85 sq. m.	¼–½ ½–1 1–2	7.1 9.3 6.2	704 234 67	1.45 0.76 0.31	486 308 (335) 216	745	95 84 49	127	0.19 0.27 0.23
G	D. Wt. = 79 kgm. Ht. = 75 in. Surface area = 2.06 sq. m.	0–1½		441	0.52	850	822		150	0.18
K	W. B. Wt. = 73 kgm. Ht. = 67¾ in. Surface area = 1.85 sq. m.	¼–½ ½–1 1–2	7.1 9.3 6.2	408 204 89.6	2.60± 1.10 0.27	157 185 (225) 332	745	98 92 90	127	0.62 0.41 0.27
K	D. Wt. = 79 kgm. Ht. = 75 in. Surface area = 2.06 sq. m.	0–1		90	0.48	188	822		150	0.80
K	H. Wt. = 57.3 kgm. Ht. = 70½ in. Surface area =	¼–½	5.9	188	0.30	627	685	124	129	0.20
K	L. B. Wt. = 61½ kgm. Ht. = 66½ in. Surface area = 1.69 sq. m.	0–½		150	0.40	375	675		127	0.34
K	J. W. Wt. = 60.5 kgm. Ht. = 67 in. Surface area = 1.68 sq. m.	0–½ ½–1 1–2		116 69 18.6	0.84 0.46 0.09	138 150 (165) 207	670		126	0.76

TABLE II—*Continued*

Penicillin species	Subject	Time period	Urine flow	Urine penicillin	Average serum penicillin	Renal clearance of penicillin: Experimental (Aver.)	Renal clearance of penicillin: Calculated from surface area	Rate of glomerular filtration: Experimentally detd. with inulin	Rate of glomerular filtration: Calculated from surface area	Ratio of glomerular filtration to penicillin clearance
		hours	*ml. per min.*	*µg. per min.*	*µg. per ml.*					
X	T. Wt. = 58 kgm. Ht. = 66¾ in. Surface area = 1.66 sq. m.	¼–½	18.8	579	0.37	1560		140		0.21
		½–1	8.5	147	0.22	668 (920)	?	144	126	0.27
		1–2	2.3	40	0.075	533				
	J. W. Wt. = 60.5 kgm. Ht. = 67 in. Surface area = 1.68 sq. m.	0–1		241	0.40	600 (800)	670		126	0.21
		1–2		202	0.20	1010				0.13
	R. W. Wt. = 53 kgm. Ht. = 64 in. Surface area = 1.55 sq. m.	½–1	2.6	76.5	0.135	567	625		117	0.21
		1–2	6.5	27.9	0.054	517 (540)				0.23
	G. Wt. = 75 kgm. Ht. = 68 in. Surface area = 1.86 sq. m.	¼–½	15.9	635	0.45	1410 ?		154		0.11 ?
		½–1	7.8	169	0.20	845 (865)	750	154	141	0.18
		1–2	4.1	287	0.084	342		69		0.20
	B. H. Wt. = 51 kgm. Ht. = 64 in. Surface area = 1.52 sq. m.	1–2	0.9	188	0.41	460				0.25
		2–3	2.7	72	0.14	534 (450)	453		115	0.22
		3–4	1.0	19	0.05	366				0.31
	S. Wt. = 67 kgm. Ht. = 69¼ in. Surface area = 1.82 sq. m.	0–1		410	0.60	700±	763		137	0.20
		1–2		157	0.19	826 (760)				0.17

time for which penicillin remained at measurable levels precluded a precise measurement of the average blood level during the period of urine collection, and the figures in Table V with respect to the renal clearance of penicillin K are of dubious significance.

In an attempt to saturate the tubular secretory mechanism, rabbits were injected intravenously with 6, 60 and 600 mgm. per kgm. of penicillin G, and blood and urine specimens were obtained for assay in the usual manner. As is shown in Table IV, after injection at 6 or 60 mgm. per kgm. the renal clearance of penicillin remained at a high level which did not vary significantly over the entire range of plasma concentration studied, and approximated the total renal plasma flow. Saturation of the tubular excretory mechanism was, however, achieved by a single intravenous injection of penicillin G at 600 mgm. per kgm., which corresponds to 60 million units in the average human adult. As is shown in Table V, in 2 rabbits so injected, with initial blood levels 5 to 7 minutes after the injection , of 2,667 and 3,200 micrograms per ml., the renal clearance of penicillin in the first hour after the injection was abnormally low (9.0, decreasing to 3 ml., in rabbit 5791, and 7 ml. per minute in rabbit 5939). These values are of the same order of magnitude as the normal rate of glomerular filtration reported by Walker and his associates (18). The renal clearance remained at this low level until the plasma concentration had fallen in 1 instance to approximately 1,500, and in the other to approximately 1,000 µg. Thereafter, the renal clearance rose, in 1 instance sharply,

TABLE III

The renal clearance of penicillin G in man after the intramuscular injection of a suspension in peanut oil and beeswax

300,000 units per ml. equals 180 mgm. per ml.

Patient	Penicillin dosage	Urine collection		Urine penicillin	Average penicillin blood level	Renal clearance of penicillin	Glomerular filtration rate*	Ratio of glomerular filtration to total renal clearance
		Time periods	Urine flow					
		minutes	*ml. per min.*	*µg. per min.*		*ml. per min.*		
A. K.		35–56	1.0	213	0.36	592	133	0.23
Ht. = 71¾ in.	1 ml. = 2.3 mgm. per kgm.	56–77	2.1	309	0.40	772	184	0.24
Wt. = 79 kgm.		77–104	2.5	245	0.42	583	187	0.32
Surface area = 2.0 sq. m.		104–166	1.6	476	0.60	793		
		166–185	1.5	1010	0.92	1097		
		185–391	0.7	311?	1.10	283		
						Experimental average *687*	*168*	*0.24*
						Calcd. from surface area *796*	*150*	
S. C.		37–56	1.6	361	0.65	555	150	0.27
Ht. = 65½ in.	0.9 ml. = 3.1 mgm. per kgm.	56–78	1.4	364	0.76	479	112	0.24
Wt. = 52 kgm.		78–96	2.1	507	0.90	563	145	0.26
Surface area = 1.57 sq. m.		96–118	2.6	383	1.00	383	111	0.29
		118–145	2.8	383	1.00	383	99	0.26
		145–240	1.2	508	1.10	462		
						Experimental average *471*	*123*	*0.25*
						Calcd. from surface area *632*	*118*	

* Experimentally determined with sodium thiosulfate.

and in 1 progressively, to reach peak values of 18 to 28 and 23 to 57 ml. per minute. As is shown in Figure 4, the rate at which penicillin G disappeared from the blood of these 2 rabbits paralleled its renal clearance. During the phase of tubular saturation, the blood level fell slowly; but as the renal clearance increased toward normal levels there was a parallel accelerating drop in the serum concentration, particularly evident in rabbit 5791.

In the latter rabbit the glomerular filtration rate and renal plasma had been determined experimentally with sodium thiosulfate and para-aminohippuric acid, 2 days before the injection of the penicillin. The observed glomerular filtration rates of 6.0 and 6.4 ml. per minute agree with the renal clearance of penicillin during the period of tubular saturation (3 to 9 ml.); while the para-aminohippuric acid clearances of 17.5 and 18.0 ml. per minute are in reasonably good agreement with the penicillin clearances of 18 to 28 ml. per minute, obtained when the plasma levels had fallen below the level of tubular saturation.

The results in a number of rabbits' experiments in which the animals received varying amounts of commercial sodium penicillin (of unknown composition with respect to penicillins F, G, K, and X), given as a single injection, are summarized in Table V. In 7 rabbits injected at dosages of 4,000, 8,000, or 150,000 units per kgm., the average renal clearances were 53, 57, 40, 28, 42, 24, and 43 ml. per minute. In these experiments the blood level varied from a peak of 45 µg. per ml. to a low of 0.044 µg.

In rabbit 5660, injected with commercial penicillin at 1,200,000 units per kgm., the renal clearance was abnormally low, with observed values of 10, 13, 5.7, and 4.9 ml. per minute in successive time periods. As had previously been found with penicillin G, the tubular secretory mechanism had been saturated by the high blood levels, which in this animal ranged from 580 to 800 µg. per ml.

DISCUSSION

1. The data here presented indicate that in both rabbits and man, penicillins F, G, and X are secreted into the urine by the kidney at a rate which corresponds essentially to their total removal from the blood reaching that organ. The normal renal clearance of penicillin so closely approximates the renal plasma flow as determined experimentally with para-aminohippuric acid that it may be used as a test of kidney function. Indeed, penicillin has

TABLE IV

The renal clearance of penicillins F, G, K and X in rabbits

Single intramuscular injection

Penicillin species	Rabbit no.	Weight	Penicillin dosage	Time of urine collection	Urine penicillin	Average serum penicillin	Renal clearance of penicillin
			mgm. per kgm.	*hours*	*μg. per ml.*	*μg. per ml.*	*ml. per min.*
F	5244	2.25	0.35	0–1	7.7	0.25	31
	5245	2.5	0.6	0–1 1–2	14.3 2.5	0.42 0.063	34 40 (37)
	5296	2.22	0.6	0–1 1–2	8.6 0.86	0.4 0.1	22 8.6
	5446	3.14	0.6	½–1	6.9	0.15	46
G	5318	2.72	0.6	0–1	30	0.27	111
	5428	2.73	0.6	½–1 1–2	10.5 2.83	0.38 0.14	28 20 (24)
	5445	3.08	0.6	½–1 1–2 2–3	10 5.75 3.8	0.18 0.11 0.07	55 52 (54) 54
	5467	2.8	0.6	½–1 1–2	12.3 3	0.2 0.038	62 79 (70)
	5159	2.37	1.5	1–2	23	0.42	55
	5187	2.24	1.5	1–2	14.7	0.85	17
	5843	2.74	6	1/12–¼ ¼–½ ½–1 1–2	1,170 384 90.5 21.6	11 3.1 1.3 0.25	106 124 69 (96) 86
	5823	2.48	60	0–1 1–2 2–4 4–6	1,813 249 66.7 7.5	20 6.3 2.65 0.35	91 39 25 (44) 21
	5791*	2.44	600	7 min.–¼ ¼–½ ½–1 1–2 2–4 4–6 6–8 8–10 10–12 12–13½	20,800 13,050 4,630 5,000 2,338 452 58 40.8 27.8 21.3	2,300 1,800 1,560 1,500 130 5.2 2.6 1.8 1.0 0.92	9.0 7.3 3.0 3.3 18 90 22 22 28 23
	5939†	2.82	600	0–36 min. 36–66 min. 66–106 min. 106–136 min. 136–166 min. 166–199 min. 199–232 min. 232–262 min.	16,667 7,310 8,890 2,600 1,891 931 415 320	2,400 1,050 350 110 40 16.3 10 6.7	6.9 7.0 23 24 47 57 42 45

TABLE IV—*Continued*

Penicillin species	Rabbit no.	Weight	Penicillin dosage	Time of urine collection	Urine penicillin	Average serum penicillin	Renal clearance of penicillin
			mgm. per kgm.	*hours*	*µg. per ml.*	*µg. per ml.*	*ml. per min.*
K	5186	2.73	0.6	0–1	8.15	0.3±	27
	5210	2.23	0.6	0–1	12.3	0.12±	100
	5242	3.16	0.6	0–1	10.1	0.35?	29
	5319	3.09	0.6	0–1	8.8	0.17±	52
	5447	2.93	0.6	0–½	9.0	0.22±	41
X	5252	2.35	0.6	1–2	2.9	0.115	25
	5258	2.11	0.6	1–2	3.6	0.09	40
	5327	2.85	0.6	1–2	5.7	0.16	36
	5449	3.03	0.6	½–1 1–2 2–3	18.3 5.6 1.66	0.265 0.11 0.04	69 51 (54) 42
	5465	2.8	0.6	½–1 1–2 2–3	2.7(?)* 2.7 1.75	0.165 0.095 0.07	17(?) 28 (23) 25

* Injected intravenously instead of intramuscularly.
† Average value questionable because of rapidly changing blood level in this period.

certain unique points of superiority over diodrast, para-aminohippuric acid, or any other substance currently used for that purpose. The plasma concentrations of the latter compounds cannot exceed a level of 3 to 5 mgm. per cent, as higher concentrations may so overload the tubular mechanism responsible for their secretion that complete extraction does not take place. On the other hand, plasma concentrations much lower than 1 mgm. per cent do not permit accurate determinations by the usual laboratory methods. In consequence, the useful range of plasma concentration varies only 5-fold. With penicillin, however, because the biological method used for assay is sensitive to as little as 1 part in 80,000,000 (0.00125 mgm. per cent), and because complete renal clearance is observed up to a minimum level of 1 mgm. per cent in man (10 µg. per ml.), and in rabbits of at least 4 mgm. per cent, there is as least a 1,000-fold range of plasma concentration within which the renal clearance of penicillin can be used as a measure of renal function in man.

2. The low renal clearance of penicillin K in man, approximately ½ to ⅕ that of penicillins F, G, or X, would at first sight imply that penicillin K is excreted more slowly than the other species. This should be reflected in a more sustained blood level. Instead, previous work in this and other laboratories (2 to 4) has shown that penicillin K disappears from the blood more rapidly than do the other penicillins. Moreover, the total urinary excretion in man has been shown to average only 30 per cent of the amount injected, as compared with recoveries for G and X of 80 to 100 per cent. The contradictions involved in a low renal clearance, a rapidly falling blood level, and a low urinary recovery may be more apparent than real. The low urinary recovery of penicillin K, and its rapid disappearance from the blood are probably referable to the fact that it is bound and inactivated by both the plasma (14, 20) and tissues (21, 22, 23) to a greater extent than is e.g. penicillin G. That combination with plasma protein may be not only quantitatively more complete, but also less freely reversible, and thus prevent the complete removal of penicillin K from the blood by the renal secretory mechanism.

3. The fact that penicillins F, G, and X have a renal clearance approximating the total plasma flow through the kidney is reflected in the rapidly falling blood levels observed after their intravenous or intramuscular injection in aqueous solution.

Attempts to modify the rate of excretion by reducing the rate of urine flow, as by restricting salt and water intake, or by administering pitressin, are physiologically unsound. As is true of other substances with maximal tubular secretion, the renal clearance of penicillin has been shown to be unaffected even by wide variations in the rate of urine flow. Such measures could modify the rate of secretion only by affecting the blood flow to the kidney.

A second suggested method of delaying the excretion of penicillin is to block its excretion by the administration of other substances excreted by the same tubular mechanism. Diodrast, para-aminohippuric acid and benzoic acid (5 to 10, 12) have all been reported as effective in this respect. The difficulty lies in the fact that these blocking substances are as rapidly excreted as the penicillin itself. To maintain an effective concentration of such blocking agents may prove no less laborious, and with some of these agents, no less costly, than to repeat the injections of penicillin or to increase the dosage.

The most effective method yet suggested of prolonging the time for which penicillins F, G, and X remain in the blood at effective concentrations is to delay its absorption from an intramuscular depot. This has been accomplished by injecting the drug as a suspension in peanut oil and beeswax (15). The absorption then proceeds at a slower rate than the excretion, and the time for which the penicillin remains in the blood in demonstrable and effectively bactericidal concentrations is significantly prolonged.

4. It is clear from the data here reported that the therapeutic efficacy of penicillin would be greatly enhanced were it possible by appropriate chemical modification to decrease its renal clearance. An antibiotic with the same bactericidal activity as

TABLE V

The renal clearance of penicillin in rabbits

Single intramuscular injection of commercial sodium salt

Rabbit no.	Weight	Penicillin dosage	Time of urine collection	Urine penicillin	Average serum penicillin	Renal clearance of penicillin
	kgm.	*units per kgm.*	*hours*	*units per min.*	*units per ml.*	*ml. per min.*
3851	2.5	4,000	1–2 2–3	8.55 2.3	0.16 0.044	53 52 } 53
3925	2.8	4,000	1–2 2–3	12.4 4.5	0.28 0.065	44 69 } 57
4100	2.64	8,000	1–2 2–3	38.1 6.9	1.05 0.16	36 43 } 40
4157	2.62	8,000	1–2 2–3 3–4	20.5 6.7 0.78	1.0 0.18 0.03	21 37 } 28 26
4132	2.43	8,000	1–2	41.5	1.0	42
4158	2.32	8,000	1–2	7.1	0.3	24
5961	3.7	150,000	¼–½ ½–1 1–2 2–3 3–4	1,408 2,432 1,360 757 596	43 45 34 19 13	33 54 40 } 43 39 46
5660	2.7	1,200,000	¼–½ ½–1 1–2 2–3	5,713 9,856 4,576 3,422	580 770 800 700	10 13 5.7 } 8.4 4.9

TABLE VI

The renal clearance of penicillins F, G, K, and X in man and in rabbits

Summary of all experiments

Penicillin species	Renal clearance, ml. per minute					
	Man				Rabbit	
	Observed clearances			Mean of all expts.	Observed clearances	Mean
	Continuous infusion	Single injection, aqueous	Single injection, POB			
F	550 900			725	31, 37, 15, 46	32
G	525	335 850	687 409	560	111, 24, 54, 70, 55, 18, 96, 44, 24, 65	56
K	272 269	225 188 627 375 165		300		
X	652	920 800 542 865 450 763		710	26, 40, 36, 54, 23	36
Commercial penicillin					53, 57, 40, 28, 42, 24, 43	41

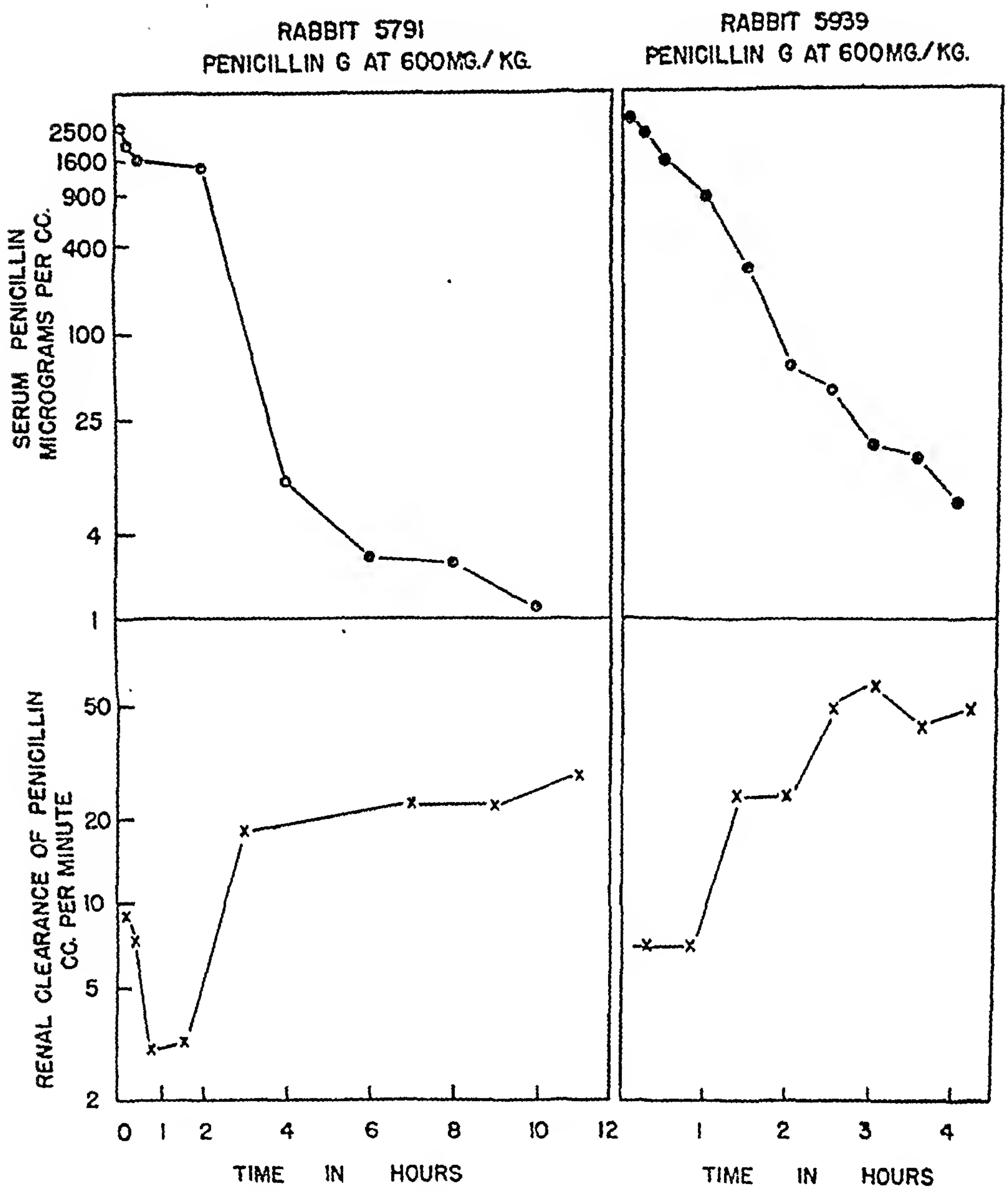

FIG. 4. THE SATURATION OF THE RENAL SECRETORY MECHANISM FOR PENICILLIN G BY A SINGLE MASSIVE INTRAVENOUS DOSE OF 600 MGM. PER KGM.

penicillin *in vitro,* but with a renal clearance in man of *e.g.,* 150 instead of 600 ml. per minute, might well be many times more active, since both the serum concentration of penicillin and the time for which an effective level was maintained would thereby be increased. The present experiments with F, G, and X indicate that the groupings present in these 3 species have no demonstrable effect on the rate of tubular secretion; and the apparently lower renal clearance of K is more than counteracted by some of its other pharmacological properties. It is nevertheless possible that derivatives of penicillin produced either by direct chemical modification or by the addition of appropriate precursors to the culture medium may have a significantly lower renal clearance than the 4 natural penicillins here studied, and a correspondingly enhanced therapeutic activity.

SUMMARY

1. The renal clearance of penicillins F, G, and X in man was found to approximate the total renal plasma flow and was approximately 4 to 5 times greater than the renal clearance of inulin or sodium thiosulfate. The penicillin clearance was independent of the absolute blood level over the entire

range 0.05 to 10 μg. per ml. and was similarly independent of the rate of urine flow.

2. The possibility is suggested that penicillin can be used in lieu of para-aminohippuric acid or diodrast as a test of renal plasma flow and renal function.

3. The renal clearance of penicillin K in man varied from ¼ to ½ that of F, G, or X. Possible explanations for this anomalous result are discussed in the text.

4. The tubular excretory mechanism was completely saturated in 2 rabbits receiving 600 mgm. per kgm. of penicillin G. In these the initial blood levels were 2,667 and 3,200 μg.; and the initial renal clearances of 3 and 7 ml. per minute corresponded to the glomerular filtration rate. As the serum concentration fell to less than the saturation level for the tubular mechanism, the renal clearances rose to normal levels of 18 to 28 and 23 to 57 ml. per minute.

5. Although no differences were found between the renal clearance of penicillins F, G, or X, the possibility is suggested that penicillin derivatives may be produced with significantly lower clearances than those of the natural penicillins so far identified, and with correspondingly enhanced therapeutic activity *in vivo*.

BIBLIOGRAPHY

1. Rantz, L. A., and Kirby, W. M. M., Absorption and excretion of penicillin following continuous intravenous and subcutaneous administration. J. Clin. Invest., 1944, 23, 789.
2. Eagle, H., and Musselman, A. D., The low therapeutic activity of penicillin K relative to that of penicillins F, G, and X, and its pharmacological basis. Science, 1946, 103, 618.
3. Coghill, R. D., Osterberg, A. E., and Hazel, G. R., The relative effectiveness of pure penicillins G, X, and K. Science, 1946, 103, 709.
4. Eagle, H., The varying blood levels afforded by penicillins F, G, K, and X in rabbits and man. J. Exp. Med., 1947, 85, 163.
5. Beyer, K. H., Woodward, F., Peters, L., Verwey, W. F., and Mattis, P. A., Prolongation of penicillin retention in body by means of para-aminohippuric acid. Science, 1944, 100, 107.
6. Beyer, K. H., Flippin, H., Verwey, W. F., and Woodward, R., Effect of para-aminohippuric acid on the plasma concentration of penicillin in man. J. A. M. A., 1944, 126, 1007.
7. Beyer, K. H., Peters, L., Woodward, R., and Verwey, W. F., Enhancement of the physiological economy of penicillin in dogs by the simultaneous administration of para-aminohippuric acid. II. J. Pharmacol. & Exper. Therap., 1944, 82, 310.
8. Beyer, K. H., Verwey, W. F., Woodward, R., Peters, L., and Mattis, P., The enhancement of the plasma concentration of penicillin in dogs by the simultaneous administration of para-aminohippuric acid. III. Am. J. M. Sc. 1945, 209, 608.
9. Loewe, L., Rosenblatt, P., Alture-Werber, E., and Kozak, M., Prolonging action of penicillin by para-aminohippuric acid. Proc. Soc. Exper. Biol. and Med., 1945, 58, 298.
10. Rammelkamp, C. H., and Bradley, S. E., Excretion of penicillin in man. Proc. Soc. Exper. Biol. & Med., 1943, 53, 30.
11. Bronfenbrenner, J., and Favour, C. B., Increasing and prolonging blood penicillin concentrations following intramuscular administration. Science, 1945, 101, 673.
12. Moehlig, R. C., and Linkner, L. S., Penicillin with pitressin tannate for delaying excretory loss; preliminary report. Harper Hosp. Bull., 1945, 3, 73.
13. Eagle, H., Relative activity of penicillins F, G, K, and X against spirochetes and streptococci *in vitro*. J. Bact., 1946, 52, 81.
14. Eagle, H., The inactivation of penicillins F, G, K, and X by human and rabbit serum. J. Exp. Med., 1947, 85, 141.
15. Romansky, M. J., and Rittman, G. E., Penicillin: 1. Prolonged action in beeswax-peanut oil mixture. 2. Single injection treatment of gonorrhea. Bull. U. S. Army M. Dept., 1944, 81, 43.
16. Goldring, W., and Chasis, H., Hypertension and Hypertensive disease. The Commonwealth Fund, New York, 1944.
17. Newman, E. V., Gilman, A., and Philips, F. A., The renal clearance of thiosulfate in man. Bull. Johns Hopkins Hosp., Sept. 1946, in press.
18. Walker, A. M., Schmidt, C. F., Elsom, K. A., and Johnston, C. G., Renal blood flow of unanesthetized rabbits and dogs in diuresis and antidiuresis. Am. J. Physiol., 1937, 118, 95.
19. Tucker, H. A., and Eagle, H., The serum concentrations of Penicillin G in man following its intramuscular injection in aqueous solution and in peanut oil-beeswax suspension. In press.
20. Tompsett, R., Shultz, S., and McDermott, W., The relation of protein binding to the pharmacology and antibacterial activity of penicillins X, G, Dihydro F, and K. J. Bact., 1947, 53, 581.
21. Richardson, A. P., Miller, I., Schumacher, C., Jambor, W., Pansy, F., and Lapedes, D., Physiological disposition of penicillin G and K in dogs. Proc. Soc. Exper. Biol. and Med., 1946, 63, 514.
22. Tepperman, J., Rakieten, N., Valley, G., and Lyon, E. W., Inactivation of penicillins G and K by liver and kidney. Science, 1947, 105, 18.
23. Clowes, G. H. A., and Keltch, A. K., On comparative results obtained with crystalline penicillins K, G,

F, and X when exposed in the Warburg apparatus to the action of liver and other mammalian tissues, blood and blood fractions, and bacteria. Reported at Antibiotics Study Section Meeting, Washington, D.C., January 31 and February 1, 1947; Supplementary report on the comparative results obtained with crystalline penicillins, particularly G and K, when exposed in the Warburg apparatus to the action of normal and preheated rat liver slices, homogenized liver, and bacteria; also normal liver with enzyme-suppressing chemical agents. Reported at Antibiotics Study Section Meeting, Washington, D.C., January 31 and February 1, 1947.

24. Jensen, K. A., Moller, K. O., and Overgaard, K., Studies on the excretion of penicillin through the kidneys and the mechanism of this process. Acta Pharmacol. et Toxicolog., 1945, 1, 184.

THE BLOOD LEVELS AND RENAL CLEARANCE IN RABBITS AND MAN OF AN ANTIBIOTIC DERIVED FROM B. SUBTILIS (BACITRACIN)

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The renal clearances of penicillins F, G, and X have been shown to approximate the total renal plasma flow (1, 2), varying between 529 and 865 ml. per minute in man, and 23 to 111 ml. per minute in rabbits (2). In consequence of that rapid excretion, the serum concentration of penicillin G decreases after its intramuscular injection in aqueous solution, at an average rate of 2 to 3 per cent of the residual penicillin per minute, and 70 to 80 per cent per hour; and it disappears from the blood even more rapidly after intravenous injection (3, 8). A therapeutic agent equal to penicillin in bactericidal activity, but with a slower rate of absorption or excretion, would provide effective levels for longer periods of time, and might be correspondingly more effective than penicillin similarly injected.

The antibiotic agent discovered by Johnson, Anker and Meleney (4) in culture filtrates of a strain of B. subtilis (Tracy), and termed by them bacitracin, possesses some of these properties. As will be here shown, it is excreted by both rabbits and man at a rate which corresponds approximately to the rate of glomerular filtration, rather than to the total renal plasma flow. In consequence, the blood levels observed after its intravenous or intramuscular injection fall off more slowly than do those of penicillin, and a given dosage provides effective levels for longer periods. The implications of these findings with respect to the therapeutic efficacy of this agent are discussed in the text.

METHODS AND MATERIALS

Bacitracin. Three different lots of bacitracin were used in these studies. The courtesy of Dr. Frank L. Meleney in facilitating their procurement is gratefully acknowledged. The 3 lots, designated in the tables as A, B–100, and PB–1, had relative gravimetric activities *in vitro* against the C–203 strain of Streptococcus pyogenes of 100, 165, and 330, respectively. Their activity in terms of the unit as defined by Meleney and his co-workers (4) was 18, 30, and 60 units per mgm., respectively. Lot PB–1 was concentrated in this laboratory from a commercial lot, B–103.

Blood specimens in rabbits were obtained by cardiac puncture, and urine specimens by bladder catheterization and irrigation.

Method of assay. A modified Rammelkamp-Rantz-Kirby method (5, 6), in which inhibition of hemolysis by the C–203 strain of Streptococcus pyogenes served as the endpoint, was used to assay the levels of the antibiotic in the blood and urine. The endpoint was not as sharp as with penicillin, and somewhat coarser interpolations were used than had proved feasible with the latter drug (2, 7). The unknown sample, in appropriate dilution as determined by a preliminary spot-assay, was distributed in amounts of 0.8, 0.64, 0.48, 0.4, 0.32, 0.24, 0.2 . . . ml., and the volumes brought up to 0.8 ml. with broth. To all the tubes were then added 0.5 ml. of 4 per cent rabbit (or human) blood broth, inoculated with 1/2,500 part of a fresh 6- to 8-hour culture of the organism in blood broth. Difficulties introduced by the occasional contaminated specimen were overcome by removing the assays from the incubator after 6 to 8 hours, when the streptococci had multiplied sufficiently to initiate hemolysis, but before they had been overgrown by the contaminant. The tubes were then allowed to stand at room temperature overnight, and the results read the next morning.

The threshold concentration for inhibition under the conditions of this assay averaged on the order of 0.01 "units" per ml., or 0.3 µg. of the impure preparation B-100. The threshold concentration of penicillin G under the same conditions was 0.015 µg. per ml.

EXPERIMENTAL

Blood levels of bacitracin in rabbits and man. Nine human subjects were injected intramuscularly with bacitracin in aqueous solution at dosages

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varying from 14 to 150 units per kgm., representing 0.8 to 5 mgm. per kgm. of the preparations used. Rabbits were injected both intravenously and intramuscularly at dosages varying from 10.8 to 1,080 units per kgm. (0.6 to 60 mgm. per kgm.). The blood levels at varying intervals thereafter are graphically summarized in Figures 1 and 2, in which the bacitracin dosages and blood levels are expressed both as units and milligrams in order to permit comparison with the average curves given by penicillin G similarly injected.

In individual rabbits, the serum concentrations at any given time were linearly related to the dosage injected; and the average curves of Figure 1 reflect the constancy of that relationship. In man, the relationship between dosage and blood level was evident, but less regular (Table I).

There was a striking difference between the rate at which bacitracin and penicillin G disappeared from the blood. In rabbits injected intravenously, bacitracin disappeared from the blood at an average rate of 50 per cent of the residual activity per hour. With penicillin G, the hourly rate of fall has previously been found to be 70 to 80 per cent after

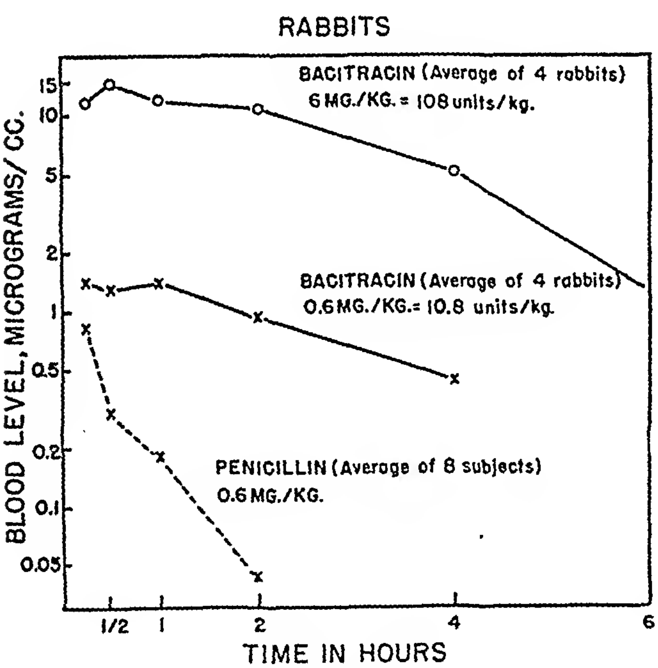

FIG. 1. THE AVERAGE BLOOD LEVEL OF BACITRACIN IN RABBITS AFTER ITS INTRAMUSCULAR INJECTION AT 6 MGM. PER KGM. AND 0.6 MGM. PER KGM. OF THE CRUDE PREPARATION A, CORRESPONDING TO 108 AND 10.8 MELENEY-JOHNSON UNITS PER KGM.

The blood levels are expressed in micrograms to permit comparison with the penicillin curve; the activity in units would be 0.018 times the levels shown in the figure.

TABLE I

Blood levels of bacitracin after its intramuscular injection in man

Subject	Dosage		Time in hours: ¼	½	1	2	4	6	8	24
			Blood levels							
	mgm. per kgm.	*units per kgm.*	*units per ml. × 10⁻²*							
W. H.	5	150		19	43	43	2.0		<1.5	
F. E.			6	19	25	33	1.7		3.0	<
J. M.	4	120		6.6	19	23	7.5	1.5	<	
T. B.	3	90	10	20	27	14	7.2	2.3	1.8	
J. P.	2	60	4.5	12.0	20	29	14	<1.5		
L. C.	1.5	45	1.8	3.3	6	3.6	1.8	<1.5		
J. A.			4.5	7.2	12	12.0	3.6	2.3		
G. H.	1.1	20	3	3.8	3.8	2.5	<			
D. K.		15	1.8	3.0	3.8	3.3	1.5			

All except last 2 patients (G. H. and D. K.) injected with same lot of material (B-100).

intramuscular injection, and even greater after intravenous injection. The qualitative difference between penicillin and bacitracin is apparent on comparing the appropriate curves in Figures 1 and 2. In rabbits, one hour after the injection of penicillin G and (crude) bacitracin in equal gravimetric dosage, the serum levels of bacitracin were 8 to 20 times greater than those of penicillin; and 2 hours after the injection, the difference was 20- to 100-fold. In man also (Figure 2 and Table I), the blood levels 2 hours after the injection of bacitracin

TABLE II

The varying periods for which bacitracin and penicillin, similarly injected, remained in the circulating blood

	Dosage	Average number of hours indicated drug remained in serum at minimum level of 1 μg. per ml.: Penicillin	Bacitracin*
	mgm. per kgm.		
Man (intramuscular)	3	2.1	6.8
	1.5	1.2	2.5, 4.6
Rabbit (intravenous)	60	1.5	9–10
	6	0.9	5.7
	0.6	0.25	1.6

The penicillin blood levels in man are average of 8 subjects (9). The penicillin levels in rabbit are from single animals only. Bacitracin levels are from the experiments of Table I and Figures 1 and 2.

* 1 μg. per ml. of crude preparations here used = 0.03 Meleney-Johnson unit.

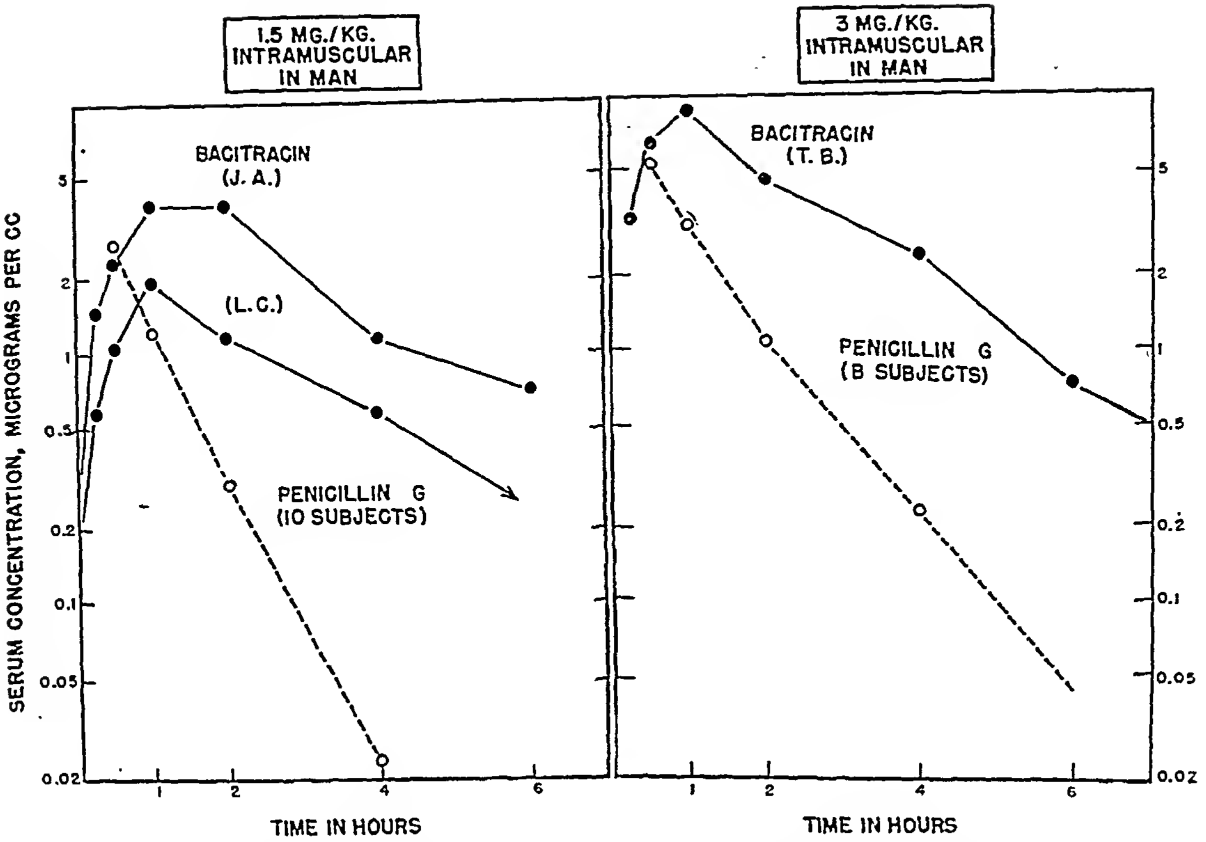

FIG. 2. THE AVERAGE BLOOD LEVELS AFFORDED BY BACITRACIN IN MAN AFTER ITS INTRAMUSCULAR INJECTION AT 1.5 MGM. PER KGM. AND 3 MGM. PER KGM. OF THE CRUDE PREPARATION B–100, CORRESPONDING TO 45 AND 90 MELENEY-JOHNSON UNITS PER KGM.

The blood levels are expressed in micrograms to permit comparison with the penicillin curve; the activity in units would be 0.030 times the levels shown in the figure.

at *e.g.*, 3 mgm. per kgm. (90 units per kgm.), were 4 times greater than the average observed with penicillin G; and the difference became progressively more pronounced thereafter.

A more important comparision between the 2 antibiotics is the length of time for which effective bactericidal levels were maintained. The pertinent data are summarized in Table II. In 2 subjects (J. A. and L. C.), injected intramuscularly with crude bacitracin B-100 at 1.5 mgm. per kgm. = 45 units per kgm., a level of 1 μg. = 0.03 unit per ml. was maintained for 2.5 and 4.6 hours (Figure 2). In 8 subjects similarly injected with penicillin G, that level was maintained for an average period of 1.2 hours (8). In subject T. B., injected with bacitracin at 3 mgm. per kgm. = 90 units per kgm., the 1-μg. level was maintained for 6.8 hours, as compared to an average of 2.1 hours in 8 patients receiving the same amount of penicillin G (8). In rabbits also, whether injected at 0.6 or 6 mgm. per kgm., a serum concentration of active material corresponding to 1 μg. per ml. was maintained 6 to 7 times longer with bacitracin than with penicillin.

Urine excretion of bacitracin. The pharmacologic basis for the marked difference in the blood levels afforded by penicillin and bacitracin is their widely different rate of urinary excretion. In 8 human subjects (Table III and Figure 3), the cumulative excretion of bacitracin in 1, 2, 4, and 6 hours averaged 17, 34, 66 and 87 per cent.[2]

[2] In 5 of the subjects listed in Table I, the total urinary excretion after 6 to 8 hours varied between 78 and 119 per cent of the amount injected, reflecting essentially com-

TABLE III

The urinary excretion of bacitracin after its intramuscular injection in man

Subject	Dosage		Time in hours						
			½	1	2	4	6	8	24
	mgm. per kgm.	*units per kgm.*	*Cumulative percentage of bacitracin excreted*						
W. H.	5	150		12.6		78.2		105.7	114.2
F. E.	5	150	2.5	8.4	25.4	51.9		78.4	95.4
J. M.	4	120	24.3	33	42.6	(125.8)*	(155)	(163.8)	
T. B.	3	90	6.5	22.3	50.5	92.9	111.4	118.5	
L. C.	1.5	45	5.4	15.4	32.5	68.2	87.7	97.3	
J. A.	1.5	45		11	29.8	59	82		
G. H.	1.1	20	6.8	19.2	29.5				
D. K.	0.82	15	4.5	12.5	30.4	47.8			
Range, per cent			4.5 to 24	8.4 to 33	25.4 to 50.5	47.8 to 92.9	82 to 111	78.4 to 118.5	
Mean per cent excreted			8.4	16.8	34.4	66.3	87	100	
Average excretion in 3 subjects given penicillin G intramuscularly				60	72	78	86		
Hourly excretion in indicated period	Bacitracin			16.8	17.6	16.0	10.3	6.5	
	Penicillin			60.0	12	3.0	4.0		

* Anomalous high results in parentheses not included in calculation of mean excretion.

The corresponding values for penicillin G have been previously reported to be 60, 72, 78, and 86 per cent (8). In the first hour, penicillin G was therefore excreted 3 times faster than bacitracin (Figure 4); after 2 hours, however, when most of the penicillin had already appeared in the urine, and its rate of excretion in the following 2 hours had fallen to 3 per cent per hour, the rate of urine excretion of bacitracin was still 16 per cent per hour, 5 times that of penicillin. The shapes of the blood curves mirror the differences in the rate of excretion of the 2 antibiotics.

In rabbits also (Table IV, and Figures 3 and 4) from 7.5 to 26 per cent (averaging 17 per cent) had appeared in the urine in the first hour, as compared with 58 per cent for penicillin G (3); but after 2 hours, bacitracin was being excreted at an hourly rate of 14 per cent, or 1.7 times greater than penicillin similarly injected. Beyond that time period, only insignificant amounts of penicillin were recovered in the urine, while bacitracin was still being excreted in appreciable quantity.

The renal clearance of bacitracin. Nine experiments to determine the renal clearance of bacitracin in man are summarized in Table V, and one experiment is graphically summarized in Figure 5. The results varied considerably from subject to subject, and even in successive time periods in the same subject. To some extent, the latter variation may have reflected incomplete voiding, since only 2 of the patients were catheterized. The average clearances in the 9 subjects varied between 105 and 283 ml. per minute, averaging 159. The corresponding average values for penicillins F, G, and X in man have been found to be 725, 560, and 710 ml. per minute (2). The renal clearance of bacitracin in man is therefore approximately ½ to ⅙ that of penicillin, explaining the more prolonged blood levels and the more sustained but initially lower rate of urinary excretion of the former.

plete excretion. In 1 subject (J. M.), however, the cumulative total of 164 per cent exceeded the experimental error of the method of assay. An even greater anomalous excretion was observed in patient J. P. In both cases, the anomalous result was due to an abnormally high excretion during the 2- to 4-hour period, as yet unexplained.

The ratio of the bacitracin clearance to the total renal plasma flow (calculated) in these 8 subjects, given in column 5 of Table V, varied between 0.15 and 0.39, averaging 0.23.

The ratio of the bacitracin clearance to the (calculated) glomerular filtration rate, given in Column 6 of Table V, varied between 0.8 and 2.04, averaging 1.23. As is indicated in the foregoing section, a significant error in clearance was introduced in 7 of the 9 subjects by the fact that the urine specimens were probably inaccurately voided. Particularly in these patients, the ratio of the renal clearance of bacitracin to the glomerular filtration rate simultaneously determined by the clearance of thiosulfate (9) was of greater significance than the absolute values of either, since an error in urine collection would not affect the ratio of the 2 clearances. This ratio of bacitracin clearance to thiosulfate clearance varied between 0.52 and 1.83, averaging 1.1.

Eight experiments to determine the renal clearance of bacitracin in rabbits are summarized in Table VI, and one experiment is shown in Figure 6. In these 8 rabbits the renal clearance of bacitracin varied between 2.0 and 7.1 ml. per minute, averaging 4.1. This is less than the glomerular filtration rate in the rabbit (10), and is to be compared with an average renal clearance in that species for penicillins F, G, and X of 34, 61, and 36 ml. per minute, with a range of 18 to 100 (2). The

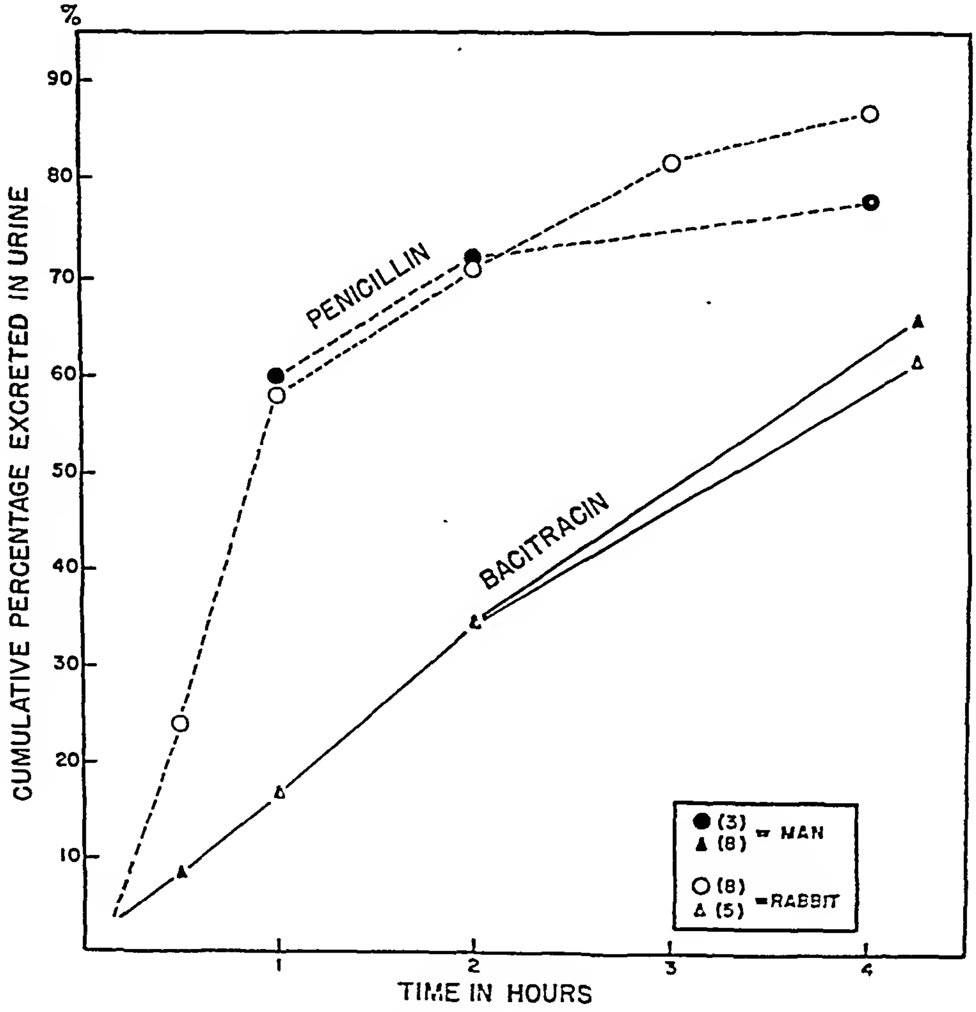

FIG. 3. THE AVERAGE CUMULATIVE EXCRETION OF PENICILLIN AND BACITRACIN IN MAN AND RABBIT

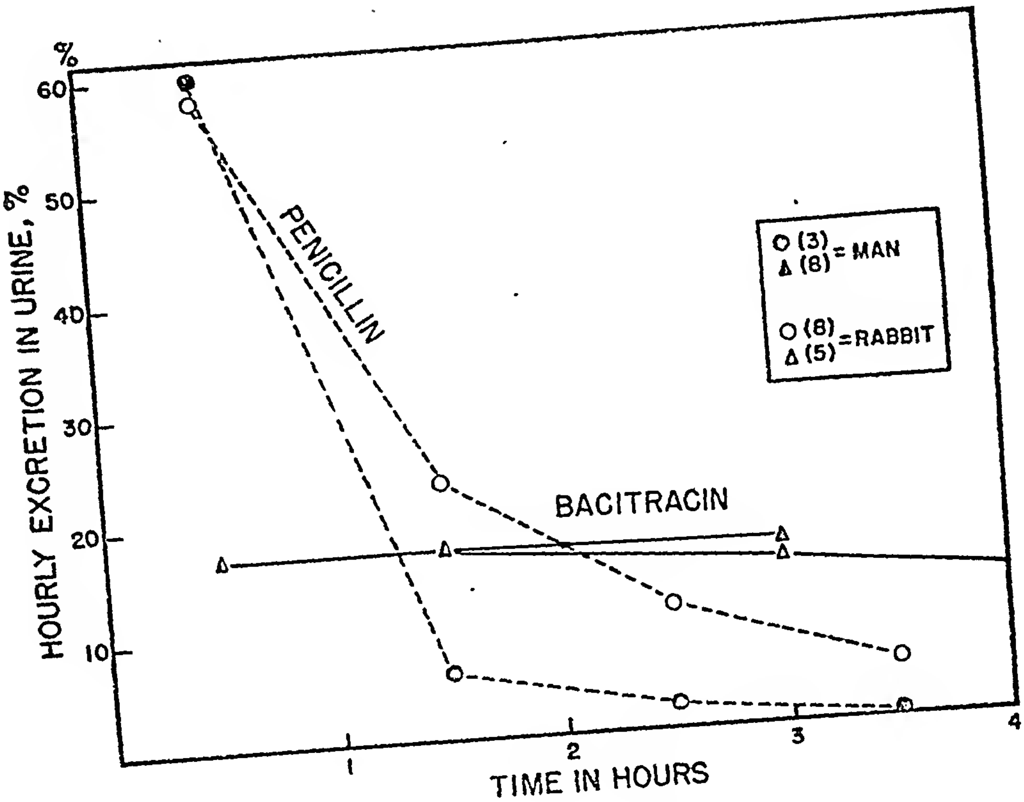

FIG. 4. THE HOURLY EXCRETION OF PENICILLIN AND BACITRACIN IN MAN AND RABBIT AFTER THEIR INTRAMUSCULAR INJECTION

TABLE IV

The urinary excretion of bacitracin and penicillin in rabbits

Route of injection	Dosage mgm. per kgm.	Dosage units per kgm.	Time in hours: ½	1	2	3	4	8	24
			Cumulative percentage of bacitracin excreted in the urine						
Intramuscular	6.0	18.0	2.2	7.5 9.0 21.1	19.5 15.0 45.5		42.8 72.5	49.2 76.8	78.6
	0.6	10.8	8.0	25.6 21.0	51.4 36.9		70.6		
			Mean	17	34		62		
Intravenous	6.0	18.0		31.4 17.5	56.0 30.0	42.3	63.7 51.1	66.5	
	.0.6	10.8		33.5	47.1		54.6		
			Mean	27.5	44.4		56.5		
Mean in 7 rabbits injected intramuscularly with penicillin G				58	71		87	87	
Hourly rate of excretion after intramuscular injection	Bacitracin			17	17		14		
	Penicillin			58	13		8		

latter values approximate the total renal plasma flow.

DISCUSSION

It is evident from the foregoing data that the antibiotic substances present in culture filtrates of

TABLE V

Renal clearance of bacitracin in man

Subject	Dosage	Average clearance bacitracin	Calc'd renal plasma flow*	Ratio bacitracin clearance to total plasma flow*	Ratio of bacitracin clearance to glomerular filtration rate: As calculated from surface area	Experimentally det'd with sodium thiosulf
	mgm. per kgm.	*ml. per min.*				
H.W	5	115	705	0.16	0.87	0.92
F. E.	5	105	665	0.16	0.8	0.52
T. B.	3	219	705	0.31	1.65	1.0
J. P.	2	170	638	0.24	1.44	0.9
L. C.	1.5	283	732	0.39	2.04	1.8
J. A.	1.5	104	672	0.15	0.82	0.7
G. H.	1.1	139	613	0.23	1.2	1.6
D. K.	0.82	141	752	0.19	1.0	1.2
Mean values		159	695	0.23	1.23	1.1

* Calculated from surface area.

certain strains of B. subtilis (bacitracin) remain in the circulating blood for significantly longer periods and at higher levels than does penicillin similarly injected. This reflects the fact that the renal clearance of bacitracin was of the same order of magnitude as the glomerular filtration rate, as compared to a renal clearance for penicillins F, G, and X, approximating the total renal plasma flow.

The mechanism whereby bacitracin is excreted by the kidney is not clear from the present data.

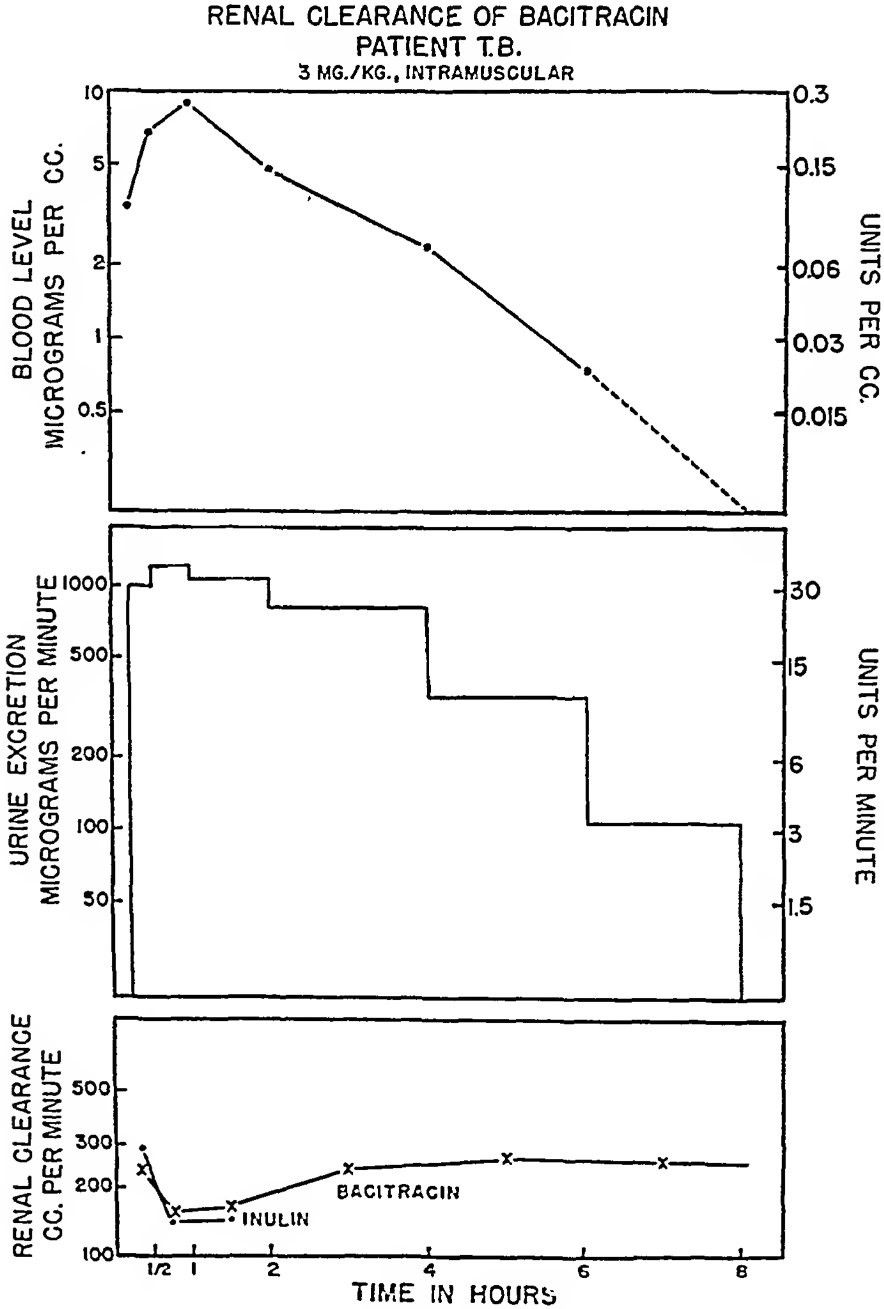

FIG. 5. ILLUSTRATING THE LOW RENAL CLEARANCES OF BACITRACIN IN MAN

Subject T. B. was injected with Preparation B-100 at 3 mgm. per kgm. = 90 Meleney-Johnson units per kgm.

TABLE VI

The renal clearance of bacitracin in rabbits

Dosage	Rabbit no.	Route of injection	Time of urine collection	Urine bacitracin	Average blood level	Renal clearance	Average
mgm. per kgm.				*mgm. per min.**	*mgm. per ml.**	*ml. per min.*	
0.6	5883	Intravenous	0 to 1 hrs.	8.6	1.7	5.0	3.4
			1 to 2	3.5	1.08	3.2	
			2 to 4	0.98	0.5	2.0	
	5897	Intramuscular	0 to 1 hrs.	5.8	1.1	5.3	5.2
			1 to 2	5.8	0.9	6.4	
			2 to 4	2.15	0.57	3.8	
	6064	Intramuscular	0 to 33 mins.	4.1	1.5±	2.7	3.5
			33 to 68	6.3	1.5	4.2	
			68 to 130	4.1	1.22	3.6	
6.0	5931	Intravenous	0 to 69 mins.	72	20	3.6	7.1
			69 to 129	65	8	8.1	
			129 to 250	18	3	6.0	
			250 to 490	4.8	0.52	3.5	
	5825	Intravenous	0 to 1 hrs.	37.9	30	1.3	2.0
			1 to 2	27	15	1.8	
			2 to 3	26.7	8.1	3.3	
			3 to 4	19	5.1	3.7	
	5912	Intramuscular	0 to 74 mins.	15	8±	2±	2.9
			74 to 131	31.5	9.6	3.3	
			131 to 253	28.7	8.4	3.4	
			253 to 495	4.0	1.5	2.7	
	5973	Intramuscular	0 to 31 mins.	12.8	5±	2.5	3.2
			31 to 65	36.2	7.4	4.9	
			65 to 130	16.5	7.6	2.2	
	6038	Intramuscular	6 to 21 mins.	31.9	7.6	4.2	5.2
			21 to 65	87.3	16.5	5.3	
			65 to 130	78.3	12.5	6.3	
			130 to 243	31.2	9.0	3.5	
			243 to 360	21.5	4.0	5.4	
			360 to 474	8.0	1.22	6.6	
			474 to 24 hrs.	0.4	?	?	

* The lot A injected contained 18 units per mgm. To translate these data to units, figures in these columns should be multiplied by 0.018.

It is possible that bacitracin is excreted only by glomerular filtration, and that the large differences obtained in the ratio of *bacitracin clearance: thiosulfate clearance*, which varied from 0.5 to 1.8, averaging 1, were due to technical factors in the collection of urine and in the assay of activity (page 919). It is, however, also possible that there is a certain amount of tubular secretion, and that nephrotoxic products known to be present in the crude bacitracin here used (11) exerted a variable inhibitory effect on the renal excretion. The degree to which bacitracin is bound by serum protein is now under study.

In any event, it is evident that the active material in the crude preparations of bacitracin here used was cleared by the kidney at a rate approximating that of glomerular filtration, while penicillin is cleared at a rate approximating the total renal plasma flow.

Against the C–203 strain of streptococcus here used, the most potent preparation of bacitracin so far available (PB–1) was gravimetrically only 1/12 as active as penicillin G *in vitro*. Unless the active material in this preparation constituted only a small fraction of its solid content, even the more favorable pharmacologic properties of bacitracin might not counterbalance its relatively low bactericidal activity against this particular strain. However, for organisms against which bacitracin has an activity *in vitro* comparable with that of penicillin, its slower excretion and more sustained blood levels might permit cure to be effected with smaller doses, or with injections administered at less frequent intervals, than is the case with penicillin.

SUMMARY

1. Three different lots of "bacitracin," antibiotic concentrates derived from culture filtrates of B. subtilis (Tracy), have been studied with respect to blood levels, urinary excretion, and renal clearance in rabbits and man.

2. The serum concentration after the intravenous or intramuscular injection of bacitracin varied linearly with dosage over a wide range. These serum concentrations fell off far more slowly, and were consistently higher, than was the case with penicillin G, similarly administered.

Two to 4 hours after injection, the serum levels of bacitracin were 10 to 50 times higher than those of penicillin similarly injected; and concentrations in excess of a given level were maintained 4 to 8 times longer.

3. Corresponding to the sustained blood levels, the rate of urine excretion of bacitracin remained at a fairly constant level for a period of 2 to 4 hours after its intramuscular injection.

(*a*) In man, the cumulative urinary excretion of bacitracin, 1, 2, 4, and 6 hours after its intramuscular injection, averaged 17, 36, 66, and 87 per cent of the amount injected. The hourly excretion in the first hour was one-third that of penicillin G; but after 2 hours, bacitracin was being excreted at a rate 5 times that of penicillin G.

(*b*) In rabbits, the cumulative totals of bacitracin excreted in 1, 2, and 4 hours averaged 17, 34, and 62 per cent of the amount injected. Initially, bacitracin was excreted at less than 1/3 the

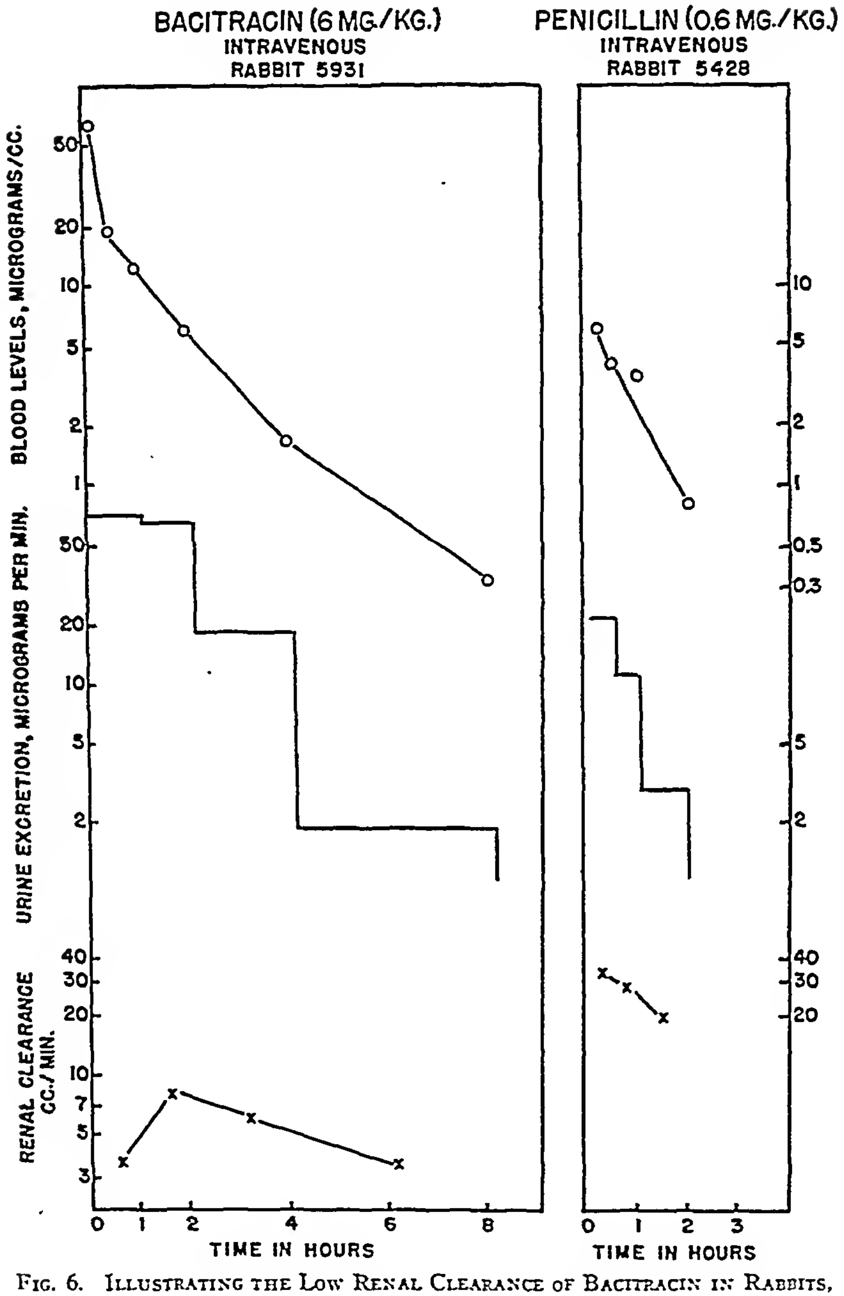

Fig. 6. Illustrating the Low Renal Clearance of Bacitracin in Rabbits, Compared to that of Penicillin

The dosages and corrections are expressed in the figure as mgm. per kgm. in order to permit comparison with penicillin. To obtain the serum concentrations and urine excretions in units, the microgram values should be multiplied by 0.018.

rate of penicillin; but after 2 hours, the rate of excretion was 1.7 times that of penicillin G.

4. The prolonged blood levels of bacitracin as compared with penicillin G, and its slower urinary excretion, are explained by its low renal clearance, which approximated the glomerular filtration rate.

(*a*) The renal clearance of bacitracin in 9 human subjects varied between 105 and 283 ml. per minute, averaging 159.

(*b*) In 8 rabbits, the renal clearance of bacitracin varied from 2 to 7 ml. per minute, averaging 4.1.

(*c*) In both man and rabbits, the renal clearance of penicillins F, G, and X for rabbits has previously been shown to approximate the total renal plasma flow.

BIBLIOGRAPHY

1. Rantz, L. A., and Kirby, W. M. M., Absorption and excretion of penicillin following continuous intravenous and subcutaneous administration. J. Clin. Invest., 1944, 23, 789.
2. Eagle, H., and Newman, E. V., The renal clearance of penicillins F, G, K, and X in rabbits and man. J. Clin. Invest., 1947, 26, 903.
3. Eagle, H., The varying blood levels afforded by penicillins F, G, K, and X in rabbits and man. J. Exp. Med., 1947, 85, 163.
4. Johnson, B. A., Anker, H., and Meleney, F. L., Bacitracin: A new antibiotic produced by a member of the B. subtilis group. Science, 1945, 102, 376.
5. Rammelkamp, C. H., A method for determining the concentration of penicillin in body fluids and exudates. Proc. Soc. Exp. Biol. and Med., 1942, 51, 95.
6. Kirby, W. M., and Rantz, L. A., Methods of measuring penicillin concentrations in body fluids. J. Bact., 1944, 48, 603.
7. Eagle, H., The inactivation of penicillins F, G, K, and X by human and rabbit serum. J. Exp. Med., 1947, 85, 141.
8. Tucker, H. A., and Eagle, H., The serum concentrations of Penicillin G in man following its intramuscular injection in aqueous solution and in peanut oil beeswax suspension. In press.
9. Newman, E. V., Gilman, A., and Philips, F. A., The renal clearance of thiosulfate in man. Bull. Johns Hopkins Hosp., 1946, 79, 229.
10. Walker, A. M., Schmidt, C. F., Elsom, K. A., and Johnston, C. G., Renal blood flow of unanesthetized rabbits and dogs in diuresis and antidiuresis. Am. J. Physiol., 1937, 118, 95.
11. Scudi, J. V., and Antopol, W., Some pharmacological characteristics of bacitracin. Proc. Soc. Exp. Biol. & Med., 1947, 64, 503.

    Scudi, J. V., Clift, M. E., and Krueger, R. A., Some pharmacologic characteristics of Bacitracin. II. The absorption and excretion of bacitricin in the dog. In press.

CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXXV. THE PERSISTENCE OF FIBRIN FILM IN TISSUE AND ITS MODIFICATION BY HEAT TREATMENT[1,2]

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(Received for publication February 7, 1947)

The fractionation of human plasma into its component proteins (1) has made possible the preparation of several new plastic materials (2, 3). These materials, fibrin film and fibrinogen plastic, are formed from native homologous proteins and have found application in surgery because of their favorable mechanical properties and their faculty of being absorbed in the body with almost no irritative reaction. The rate of absorption and the persistence time, however, depend on the modifying treatment to which they have been exposed. In its application as a dural substitute (4), thus far its most extensive clinical use, heat-sterilized fibrin film persists for periods of several months. Observations on unheated fibrin film, on the other hand, have shown much more rapid absorption. For certain uses, such as in peripheral nerve suture (5) or in tendon repair, neither of these extremes seemed desirable; the heat-sterilized film evoked too great tissue reaction, while the untreated film disappeared too rapidly to be effective.

The present study was undertaken to obtain exact information on the rate of absorption of fibrin films which had been exposed to varying degrees of heat treatment and to find, if possible, conditions which would allow fixing the persistence time to meet a given surgical need. Some quantitative measurements on the extent of gross tissue reaction to the implant are also reported. Observation on the histological sequence involved in the absorption of heat-sterilized films (6) and plastics[3] have already been made by Bailey, and a preliminary report of some of these findings has already been presented (7). Although primarily concerned with the absorption of fibrin films, some observations on the absorption of implanted fibrinogen plastic, and the tissue responses to its presence, are also reported.

Measurements were also made to determine the resistance of variously heated fibrin films to tryptic digestion. Proteolytic digestion has previously been used (8, 9) as a measure of the absorbability of surgical gut. Although the 2 processes are qualitatively similar, important quantitative differences may obtain. The absorption of fibrin film *in vivo* is compared to its tryptic digestion *in vitro*.

MATERIALS AND METHODS

Fibrin film was prepared from human fibrinogen and thrombin (10). Some films were made aseptically from solutions which had been previously sterilized by filtration; in others, unsterile materials were used and the films were subsequently sterilized by heat treatment. Fibrinogen plastic containing 67 per cent of glycerol as plasticizer was prepared by heating at 100° C. under pressure for 15 minutes. The types of material used, according to thickness and extent of heat treatment, were as follows:

A. Aseptically prepared fibrin films of 4, 7, and 11 mgm. fibrin per sq. cm.
   1. Unmodified.
   2. Heated in glycerol at 100° C. for periods of ½, 1, 3, and 10 minutes.
B. Heat-sterilized fibrin films[4] of 17 mgm. fibrin per sq. cm.

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[1] This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] This paper is Number 58 in the series "Studies on the Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

[3] Bailey, Ford, and Hawn. A report to the Proctor Fund, Harvard University, September 1, 1942. This study of the histological response to a series of implanted fibrinogen plastics was begun early in 1941.

[4] Fibrin film was at first sterilized in this manner but it was subsequently found possible to sterilize it by direct

1. Autoclaved under glycerol for 15 minutes at 120° C.
2. Heated in glycerol for 1 minute at 155° C.

C. Fibrinogen plastic of 13 mgm. dry weight per sq. cm., sterilized by autoclaving under glycerol for 15 minutes at 120° C.

Squares (1 cm. by 1 cm.) of these materials were implanted in rabbits both subdermally over the femorococcygeal muscle and intermuscularly between that muscle and the adductor group. The implants were made in normal and burned[5] tissues; however, results proved identical in both cases. Furthermore, it was observed that absorption proceeded similarly irrespective of whether implantation was subdermal or intermuscular. Consequently, the observations on these 2 situations are not treated separately, but have been combined in the discussion of results.

The implants were anchored at each corner with a loop of silk thread. They were removed at intervals and the residual fibrin was determined gravimetrically by soaking in distilled water, drying at 110° C., and weighing to 0.1 to 0.2 mgm. There was a variability of 0.2 to 0.5 mgm. in the initial weight of individual pieces and the initial weight was taken as the average of a series of 6 squares. In the late stages of absorption the difficulty in locating and removing the remaining fragments of fibrin introduced an additional error.

In measuring tryptic digestion a 1 per cent solution of a commercial trypsin in phosphate buffer of pH 7.0 and ionic strength of 0.15 was clarified by centrifugation and filtration. It was allowed to act at 38° C. for various lengths of time, and the residual fibrin was determined as

exposure to steam (3, 11) and material for clinical use is now prepared in this manner. In use as a dural substitute the persistence time and tissue reaction to the 2 types are similar (4).

[5] In one series implants were introduced into areas which had been injured by burning in order to clarify the observation that fibrin films used therapeutically on burns often disintegrated much more rapidly than in other situations. Note reference (12).

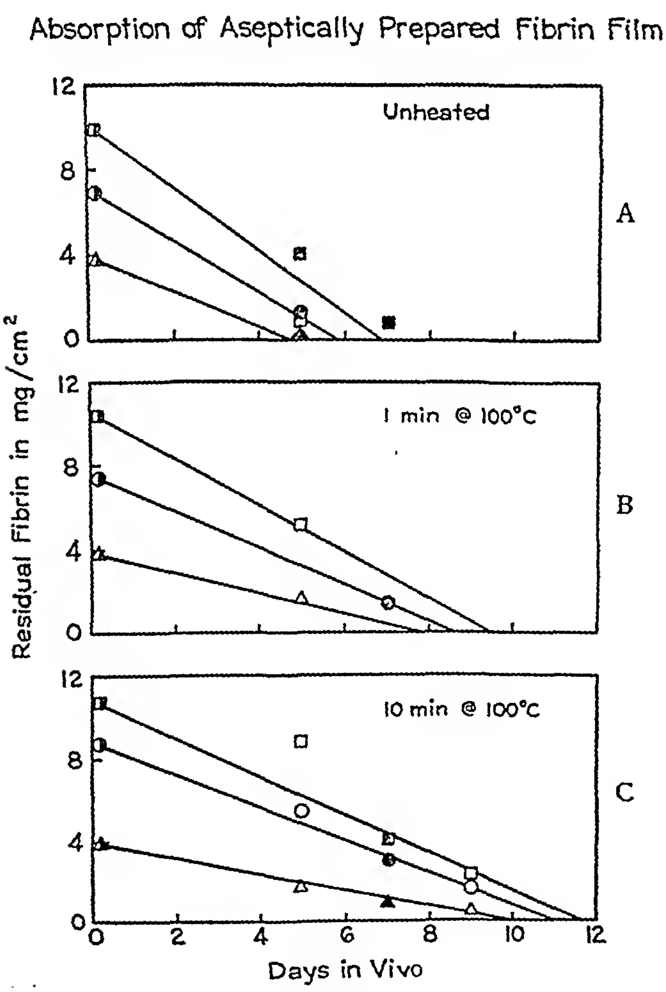

Fig. 1. The Absorption of Unheated and Moderately Heated Fibrin Films

The symbols represent different initial film weights. Open symbols indicate subdermal implantation; closed symbols, intermuscular.

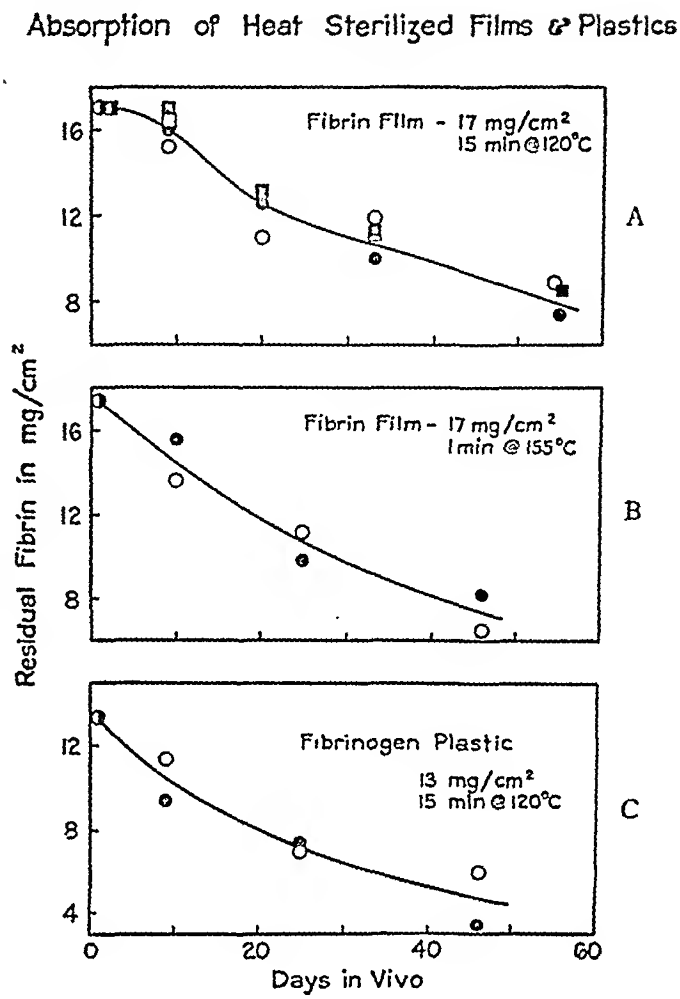

Fig. 2. The Absorption of Heat-Sterilized Films and Plastics

Open circles, subdermal implantation; closed circles, intermuscular. Squares indicate implantation in burned tissues.

above. This trypsin (Cenco) required twice as long as standard trypsin (U.S.P.) to digest heat-sterilized fibrin film. Measurements of tensile strength were made using a simple spring balance. Pieces of film 1 by 6 cm. and of a single weight, 16 mgm. per cm^2., were used in these experiments.

RESULTS

The experimental results are summarized in Figures 1 and 2 and show the progressive absorption of the fibrin following implantation. The unmodified films are absorbed most rapidly and the heat-sterilized films most slowly; the moderately heated films lie between these extremes. In presenting the data on unmodified and moderately heated films the course of absorption has been represented by a straight line whose slope gives the absorption rate and whose intercept is the persistence time. This follows the convention of Jenkins and Hrdina (13) in their studies on the absorption of surgical gut. It should be noted, however, that histological examination has revealed small fragments of fibrin after considerably longer periods than the persistence time as indicated by this method (5). The course of absorption of the heat-sterilized materials cannot be represented by a straight line since the absorption rate clearly falls off with time.

Unmodified film. Complete absorption of all the unmodified films (4, 7, and 11 mgm. per cm.^{2}) occurs in less than 9 days (Figure 1A). The absorption rates vary from 0.85 to 1.45 mgm. per day with increasing thickness of film. Even at the earliest time of examination (5 days) the film was very weak and could not be removed in a single piece.

Heat-sterilized film. The absorption curves shown by fibrin film heated for 15 minutes at 120° C. and for 1 minute at 155° C. (Figures 2A and B) are very similar. They have initial slopes (or absorption rates) of 0.24 to 0.35 mgm. per day but level off to 0.12 to 0.14 mgm. per day after 40 days in the animal. In the single animal examined after a longer period (81 days) considerable amounts of film still remained. In contrast to unmodified film, heat-sterilized film retained more of its tensile strength and could usually be removed in a single piece.

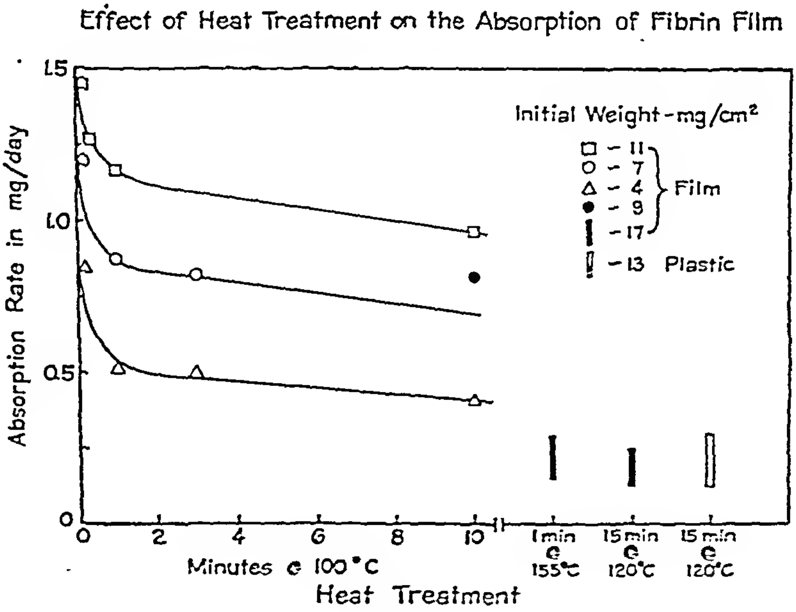

FIG. 3. THE EFFECT OF HEAT TREATMENT ON THE ABSORPTION OF FIBRIN FILM

Ordinate, rate of absorption in mgm. of fibrin per day (*i.e.*, the slopes of the curves in Figures 1 and 2); abscissa, degree of heat treatment. In the strongly heated materials whose absorption rate changed with time the range of values has been shown by the height of the symbol.

Moderately heated film. The course of absorption of films subjected to moderate heat treatment (Figures 1B and 1C) was intermediate to those of the unmodified and the heat-sterilized films. Although persisting for longer times, films heated at 100° C. for ⅓, 1, or 3 minutes resembled the unheated films in their fragmentation and rapid loss of tensile strength. On the other hand, those heated for 10 minutes behaved more like the heat-sterilized films and retained their original form during most of the absorption period. The effect of heat treatment on the absorption rates of these films has been summarized in Figure 3 and it may be seen that the 3- and 10-minute films have quite comparable absorption rates, roughly ½ that of the unmodified film, despite this marked difference in mechanical properties.

Tissue reaction to the implant. In the unmodified and moderately heated films, with their shorter persistence times, gross encapsulation by fibrous connective tissue could not be detected. In contrast, the absorption of heat-sterilized films, with their extended persistence times, always was accompanied by the development of a firm fibrous capsule. Although undoubtedly preceded by a histological sequence of events (5, 6), this capsule was grossly evident as a well-organized structure only after 10 days following implantation. It was observed to increase in thickness during the following 20 days. The capsule was easily separated from the surrounding loose connective tissue and completely enveloped the film. The film, in turn, lay free within the capsule and could usually be removed in a single piece. Internally the capsule was characterized by a shiny smooth surface having the appearance of a serous lining. Since it could be so easily separated from the surrounding tissue and the enclosed film, quantitive estimates of the response of the host to the foreign body could be obtained. The dry weight of the capsule was measured in a manner similar to that used for the film.

Figure 4 pictures the progressive growth of the capsule. The curve is characterized by a rapid increase during the 10- to 25-day period followed by a leveling off at about 10 mgm. during the succeeding 30 days. This value may be more fully appreciated when it is compared to the dry weight of the connective tissue sheet normally occurring at the implantation site which is of the order of 1 mgm. per cm². A single pair of measurements after 81 days suggests that there is a reduction in

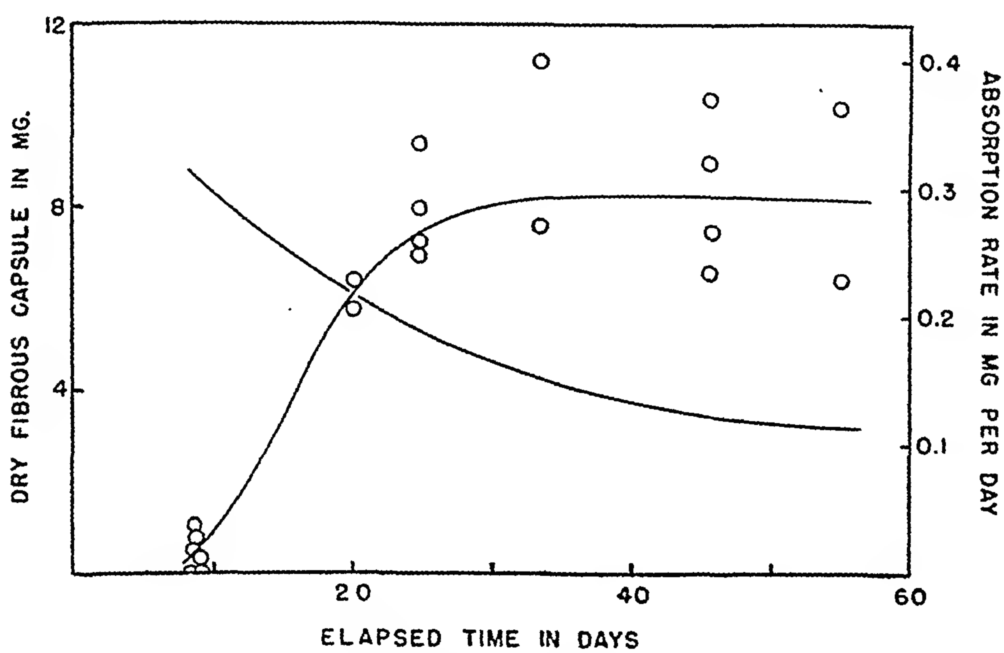

FIG. 4. THE ENCAPSULATION OF IMPLANTED HEAT-STERILIZED FIBRIN FILM AS A FUNCTION OF TIME

Points represent the dry weight of the capsule surrounding a 1 cm. by 1 cm. piece of film. The descending curve which shows the falling off in the rate of absorption with time derives from the slopes of the average curves in Figure 2.

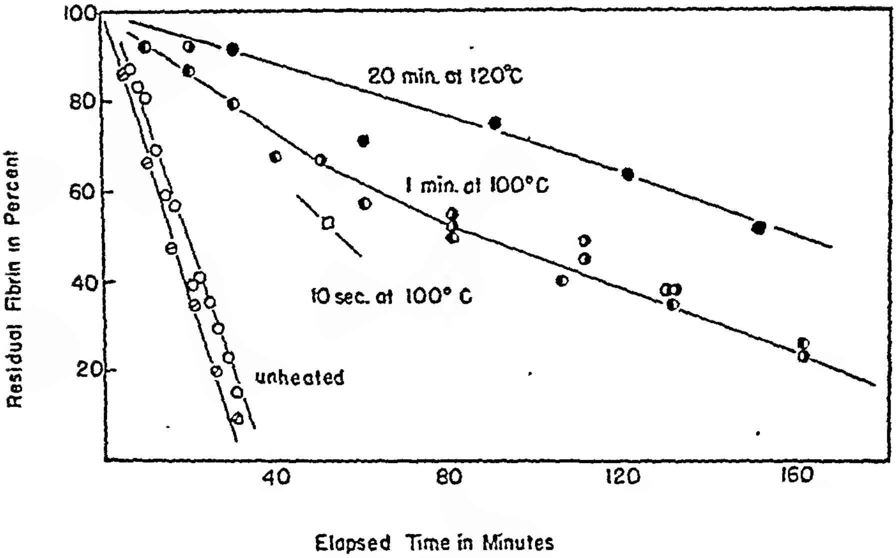

FIG. 5. THE COURSE OF TRYPTIC DIGESTION IN VARIOUSLY HEATED FIBRIN FILMS

Symbols represent different heat treatments as indicated. Temperature, 38° C.; pH, 6.9; ionic strength, 0.15; fibrin film, 12 mgm. per sq. cm., plasticizer not removed before heating.

size of the capsule as absorption of the fibrin nears completion. In these 2 instances the weights were 2.6 and 6.4 mgm. or about ½ the average value between 30 and 50 days.

Fibrinogen plastic. The course of absorption of heat-sterilized fibrinogen plastic is entirely comparable to that of heat-sterilized fibrin film. This is shown in Figure 2C where the progressive falling off of the absorption rate is clearly seen. The plastic also became surrounded by a thick fibrous capsule with a dry weight of about 10 mgm., but it retained less strength than the film, particularly in the latter stages of absorption where it fragmented badly. After 81 days only traces of the plastic could be detected.

Tryptic digestion of fibrin film. Results on the digestion by trypsin of variously heated fibrin films are summarized in Figure 5. The data are well represented by straight lines with the exception of the film heated for 1 minute at 100° C. which disappears more rapidly during the first few minutes. Under these conditions the unmodified film shows a half digestion time of 16 to 18 minutes. A very brief exposure to heat (10 sec. at 100° C.) increases this half time 3-fold, to 54 minutes. Intense heat treatment, sufficient for sterilization, extends the half digestion time only by another factor of 3, to 100 minutes.

During digestion the breaking strength of the film progressively decreases. As is shown in Figure 6 this loss in strength is in general proportional to the loss in fibrin. This is in contrast

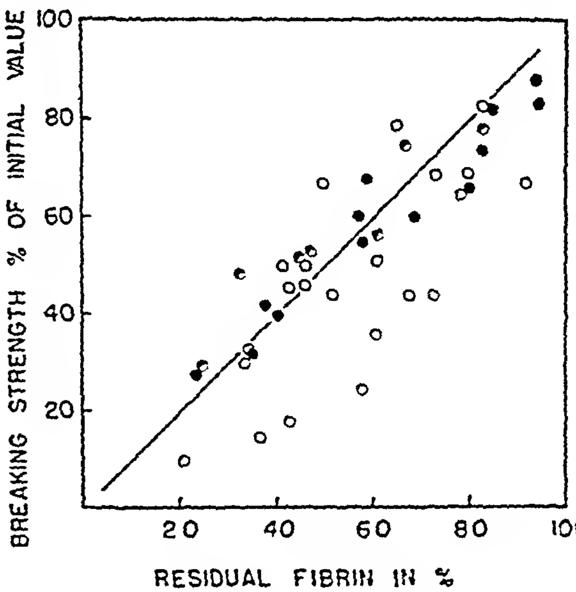

FIG. 6. THE BREAKING STRENGTH OF FIBRIN FILM UNDERGOING TRYPTIC DIGESTION AS A FUNCTION OF THE AMOUNT OF RESIDUAL FIBRIN

Symbols represent different series of measurements all performed with unheated film of initial weight of 16 mgm. per sq. cm.

to observations on the loss of tensile strength *in vivo*. The considerable scatter of the points reflects the dependence of this measurement on flaws in the film. The percentage of fibrin in the wet film becomes lower during digestion; thus the wet weight is reduced more slowly than the dry weight. This emphasizes the importance of using the latter as the measure of the proteolysis.

DISCUSSION

The wide range of absorption rates shown by the various types of film is seen in Figure 3. The effect of heating is to increase progressively the resistance of the film. However, at 100° C., the larger portion of this effect is obtained within the first minute and further heating, even for 10 times this period, makes the film only slightly more resistant. However, this additional treatment does increase the strength of the residual film. At the higher temperature of 120° C. the absorption rate is reduced to ⅕ that of the unmodified film.

Upon comparing films of different initial weight though of similar treatment (see Figure 1), one observes that the greater the thickness the greater the absorption rate. Indeed, the absorption rate in *mgm. per day* is roughly proportional to the initial weight. However, this means that the persistence time and the absorption rate in *per cent per day* should be nearly independent of the initial weight. Reference to Figure 1 shows the former to be true since, with a 3-fold difference in initial weights, the persistence times vary by a factor of only 1.2 to 1.3. This proportionality between the initial weight and the absorption rate suggests that proteolysis is occurring throughout the film rather than only at the surface. In the latter situation we would have expected different weights of film to show proportional persistence times and the same absorption rate in mgm. per day.

In this study only one thickness of heat-sterilized film was used so that similar deductions cannot be made regarding this material. However, in film sterilized by direct exposure to steam (11), tryptic digestion is more rapid in the thinner pieces. This would indicate that the enzyme is acting at the surface and that in this material the heat-imparted resistance could be accounted for, at least in part, by exclusion of the enzyme from the film interior.

In heat-sterilized film the absorption rate falls off steadily with time and the range of the changing values has been represented in Figure 3 by a vertical line. In Figure 4 this decrease in the rate of absorption is compared to the increasing encapsulation and it may be seen that there is a fairly good inverse correspondence between these 2 variables. This would suggest that this progressive decrease in the rate of absorption as these films remain in the body may be due merely to the formation of the fibrous capsule and not to any characteristic of the absorptive process.

The absorption of a protein structure in the body and its digestion *in vitro* both represent enzymatic proteolysis, and the 2 processes are therefore qualitatively similar. However, such enzymes may be quite specific and are very sensitive to the conditions under which they act (14). Extrapolation from an enzyme in one situation to a different enzyme in another situation should be made cautiously. In studies of the peptic digestion of collagen sutures Jenkins and Hrdina (9, 13) found, in general, a satisfactory correlation between the loss of strength *in vitro* and *in vivo*, but they noted that discrepancies sometimes occurred in which a suture, satisfactory by *in vitro* standards, disappeared too rapidly in the body.

TABLE I

Treatment	Time for half proteolysis			
	In vitro		*In vivo*	
	min.	heated / unheated	days	heated / unheated
Unmodified	19		3.5	
10 sec. at 100° C.	54	2.9		
1 min. at 100° C.	84	4.4	4.6	1.32
10 min. at 100° C.			5.8	1.65
15 min. at 120° C.	156	8.2	48	13.7
1 min. at 155° C.			38	10.8

A comparison of the effect of heat treatment on the digestion of fibrin film *in vivo* and *in vitro* reveals a similar picture; heat treatment increases the persistence in both situations but the quantitative relations are quite different. The half persistence times as measured by the 2 methods are summarized in Table 1, and it is seen that slight and moderate heat treatment prolongs the tryptic digestion time much more than the persistence in

the body. On the other hand, in the heat-sterilized films the change is of the same order of magnitude.

The changes in the physical and chemical properties of fibrin film which accompany heat treatment have been carefully studied (11). The heated film has a higher water-equilibrated fibrin content, a lower opacity, a higher tensile strength, a lower swelling index in acid and alkali and a lower permeability. All of these physical changes may be attributed to the formation of additional permanent cross links in the fibrin network to yield a denser, more rigid structure. The decreased permeability which results may be of particular importance in increasing the resistance of the heated film to proteolytic enzymes both *in vitro* and *in vivo*. Thus, although the heat-sterilized film is completely impermeable to hemoglobin, for example, this protein passes freely through the unmodified film. If the action of the enzyme were thus restricted to the outer surface of the film, it would result in a much slower rate of proteolysis. Of course, the increased cross linking *per se* may also increase the resistance to enzymes. The chemical changes induced by the heat treatment, which are most strikingly shown in the change in affinity for acid and basic dyes, are undoubtedly associated with the physical changes and may be also involved in the increased persistence.

SUMMARY

The course of absorption of variously heated fibrin films, after implantation in rabbits, has been studied. A quantitative measure of absorption was obtained by measuring the actual weight of residual fibrin after different lengths of implantation. A measure of the tissue reaction to the implant was obtained by measuring similarly the weight of the surrounding fibrous capsule.

Persistence times ranged from less than 5 days for unmodified fibrin film to more than 80 days for heat-sterilized films. Intermediate persistence times were obtained by milder heat treatment. A heavy encapsulation of heat sterilized film occurred between 10 and 30 days following implantation.

The resistance of these variously heated films to tryptic digestion was measured and the responses *in vivo* and *in vitro* compared. The effect of heat treatment showed qualitative similarity but important quantitative differences were noted.

BIBLIOGRAPHY

1. Cohn, E. J., Blood proteins and their therapeutic value. Science, 1945, 101, 51; Blood and blood derivatives. American Scientist, 1945, 33, 61.
2. Ferry, J. D., and Morrison, P. R., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XVI. Fibrin clots, fibrin films, and fibrinogen plastics. J. Clin. Invest., 1944, 23, 566.
3. Ferry, J. D., and Morrison, P. R., Fibrin film and other plastic products from human plasma. Ind. and Eng. Chem., 1946, 38, 1217.
4. Ingraham, F. D., Bailey, O. T., and Cobb, C. A., Jr., The use of fibrin film as a dural substitute and in prevention of meningocerebral adhesions; further studies and clinical results. J. A. M. A., 1945, 128, 1088.
5. Singer, M., The combined use of fibrin film and clot in end-to-end union of nerves; experimental study. J. Neurosurg., 1945, 2, 102.
6. Bailey, O. T., and Ingraham, F. D., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXII. Fibrin films in neurosurgery, with special reference to their use in the repair of dural defects and in the prevention of meningocerebral adhesions. J. Clin. Invest., 1944, 23, 597.
7. Morrison, P. R., and Singer, M., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XVII. A note on the absorption rates of fibrin films in tissue. J. Clin. Invest., 1944, 23, 573.
8. Kraissl, C. J., and Meleney, F. L., A method for determining the time of catgut digestion in vitro. Surg., Gynec. & Obst., 1934, 59, 161.
9. Jenkins, H. P., and Hrdina, L. S., Absorption of surgical gut (catgut). II Pepsin digestion tests for evaluation of duration of tensile strength in the tissues. Arch. Surg., 1942, 44, 984.
10. Ferry, J. D., and Morrison, P. R., Preparation and properties of serum and plasma proteins. IX. Human fibrin in the form of an elastic film. J. Am. Chem. Soc., 1947, 69, 400.
11. Ferry, J. D., Singer, M., Morrison, P. R., Porsche, J. D., and Kutz, R. L., Preparation and properties of serum and plasma proteins. X. Modification of the physical and chemical properties of fibrin film by heat treatment. J. Am. Chem. Soc., 1947, 69, 409.
12. Hawn, C. v. Z., Bering, E. A., Jr., Bailey, O. T., and Armstrong, S. H., Jr., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XIX. A note on the use of fibrinogen and thrombin in the surface treatment of burns. J. Clin. Invest., 1944, 23, 580.
13. Jenkins, H. P., and Hrdina, L. S., Absorption of surgical gut (catgut). I. The decline in tensile strength in the tissues. Arch. Surg., 1942, 44, 881.
14. See, for example, Northrop, J. H., Crystalline Enzymes. Columbia Univ. Press, New York, 1939.

STUDIES ON EXPERIMENTAL PHOSGENE POISONING. III. OXYGEN THERAPY IN PHOSGENE-POISONED DOGS AND RATS[1]

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Clinical opinion of the value of inhalational oxygen therapy in "lung irritant" casualties in the first World War was uniformly high (1 to 4). The use of oxygen in subsequent accidental poisonings has confirmed that evaluation (5 to 12) and led to extension of the original views to *pure* phosgene poisoning. The relief usually afforded the patient appears to have been impressive. Objective improvement consisted of disappearance of the cyanosis and clearing of the sensorium; the tachycardia was sometimes relieved, but the dyspnea generally was not (2). Therapy with 100 per cent oxygen has been reported to reduce cough and relieve the sensation of a constricted chest (8, 9). For these reasons, oxygen therapy is the chief measure prescribed for phosgene poisoning in official manuals and treatises on this subject. Further substantiation is necessary for other claimed benefits of oxygen therapy, such as inhibiting the development of severe edema, shortening the duration of illness, and diminishing dilatation of the right side of the heart (1, 8, 13, 14).

While it is generally agreed that oxygen therapy is clinically indicated in this type of pulmonary edema, no data have been found which permit statistical confirmation of the ultimate benefits of such therapy. A close examination of the literature brought out the following: (*a*) The clinical prognosis must have been the basis for claims of its life-saving value, but experience with poisoned animals has shown the fallibility of prognosis. (*b*) The average mortality attributable to phosgene in the A. E. F. of World War I was very low, less than 2.5 per cent of gas casualties (15). This low mortality rate automatically emphasized symptomatic relief rather than survival as the criterion of value. (*c*) At the time when the original observations were made, oxygen was administered by methods the best of which (Haldane's reservoir mask) provided concentrations of less than 60 per cent; the nasal catheter and funnel-over-the-face techniques were commonly used and the regimen varied from a few breaths at intervals to continuous use for symptomatic relief of cyanosis (1 to 3). (*d*) The true value of oxygen therapy in phosgene casualties is difficult to assess because it was but one of several drugs or procedures employed. (*e*) In accidental poisonings, deaths have been recorded despite apparently effective methods of oxygen administration (10, 11).

The previously reported data on oxygen therapy of experimentally poisoned animals contradict the clinical evaluation. Underhill (16) found that survival of poisoned dogs was not significantly improved by residence in 50 per cent oxygen for the first 72 hours after gassing, although symptomatically the dogs seemed better and the arterial and venous oxygen saturations were temporarily raised. Meek and Eyster (17) noted that the majority of an unspecified number of dogs lived 48 to 72 hours in 40 to 60 per cent oxygen, instead of the average of 16 hours in air. Dumoulin and Charlier (18) reported that 50 per cent oxygen did not increase the survival of phosgene-poisoned rats, while continuous stay in 90 per cent oxygen resulted in a still higher ultimate mortality. Soulie (19) confirmed these results and emphasized the similarity of survival rates during the first 24 hours of treatment, during which oxygen poisoning is unlikely.

Additional information was therefore necessary as to whether oxygen therapy improved the survival rate in phosgene poisoning and, if so, what were the optimum conditions of administration. Because omission of therapy in accidental poison-

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

ing might lead to loss of life, the problem was necessarily studied in animals, although it was recognized that the pathologic physiology and resistance to anoxia in the dog and rat might differ from that of man. The main experiments were patterned after Underhill's (16), using an L(CT) 60 to 80 of phosgene. This mortality was chosen as being most favorable for demonstrating a beneficial effect from oxygen therapy. Additional experiments with low oxygen tensions were carried out to test the possibility discussed by Drinker (20) that anoxia might contribute to the development of pulmonary edema, or further it in the sense of a vicious circle.

MATERIALS AND METHODS

The dogs were healthy adult mongrels, weighing 6 to 12 kgm., used after an isolation period had shown them to be free of respiratory infection. The rats were young adults, obtained directly from the Wistar Institute. The dogs in fours or sixes were exposed to a mixture of air and pure phosgene in a 850-liter gassing chamber, operated dynamically at a flow of 800 liters per minute. The mean concentration of phosgene and standard deviation were 0.275 ± 0.0045 mgm. per liter by analysis, and the duration of exposure was 30 minutes. The rats were similarly gassed in groups of 20 or 40, but for the shorter time required to give the desired mortality. Selection of gassed animals for oxygen therapy was by lottery.

Oxygen therapy was administered to the dogs by keeping them in a closed chamber of 964 liters capacity, the atmosphere of which circulated at the rate of 935 liters per minute. During circulation the atmosphere passed over a cooling radiator at 0° C., and trays of soda-lime and $CaCl_2$ in that order. By frequent sampling or automatic regulation the atmosphere was controlled to the following levels: (*a*) Humidity was less than 40 per cent, average 32 to 34 per cent. (*b*) CO_2 was less than 0.5 per cent, average about 0.2 per cent by Haldane analyzer. (*c*) Temperature was room temperature $\pm 5°$ F. (*d*) The oxygen concentration, determined by the Scholander-Roughton method or the Pauling meter, was put up by flushing the chamber with oxygen to the desired level, which then was maintained by a Pitot type injector system. Inflow of oxygen or oxygen-air was in large excess of the requirements of the animals. Three concentrations of oxygen were employed, 95 per cent, 80 per cent, and 40 per cent.[2]

Six or 8 gassed dogs, depending on size, were placed in the chamber and kept there until death, or for 72 hours. Oxygen therapy was not begun until all the animals of an experiment had been gassed and hence with different animals there was an interval of 20 minutes to 2½ hours between the end of gassing and beginning of oxygen therapy. At the end of 72 hours in oxygen the survivors were brought immediately into room air. By means of sleeved ports which permitted access into the chamber without loss of oxygen concentration, hematocrit determinations, heart rate, respiratory rate, and other clinical observations were made routinely. The animals were supplied with water, Purina checkers, and a protein digest in water at all time. Dead animals were removed from the chamber for immediate autopsy. In 2 experiments gassed rats were placed in barricaded cages in the oxygen chamber with the dogs given 95 per cent oxygen therapy.

Experiments on rats in low oxygen mixtures were carried out using either a 67-liter chamber, or the large chamber; both were operated dynamically by means of a continuous inflow of mixtures of air, nitrogen, and carbon dioxide at rates of 2 to 5 liters per minute. The rats were placed in these chambers at once after gassing and kept there for varying periods. They were unrestricted and had access to food and water. The controls remained in similar cages in room air in most experiments, but in 2 experiments the factor of air movement over the rats was controlled by placing the gassed controls in another similar chamber and passing air through it.

Oxygen toxicity control studies

In view of the probability that oxygen poisoning might be superimposed on phosgene poisoning when 80 per cent and 95 per cent oxygen were used, a series of experiments was designed to assess the effect of oxygen alone.

Three of 21 normal dogs died after 47 to 48 hours in 95 per cent oxygen, while 4 more died after 52, 59, 63, and 89 hours in this atmosphere. One animal survived 116 hours and when sacrificed after 1 hour in room air showed only slight pulmonary edema and congestion, although markedly dyspneic. This dog contrasts with another which died an acute anoxic death upon being removed from the oxygen chamber after 48 hours. Eleven of the 21 dogs were sacrificed at the end of 48 hours in oxygen; these animals showed edema of the larynx, some pulmonary congestion, and minimal pulmonary edema; and the lung-to-body weight ratios were slightly beyond the upper limits of normal. One dog lived indefinitely following 48 hours residence in 95 per cent oxygen.

Eight dogs were confined in 80 per cent oxygen for 72 hours and none died. Four, sacrificed at the end of that period, showed mild pulmonary congestion and slight pulmonary edema, and the lung-to-body weight ratios again were at the upper limits of normal. The remaining 4 dogs survived without apparent difficulty.

Adult rats appeared to show somewhat greater susceptibility to oxygen poisoning: 18 of 20 rats died after 36 to 65 hours in 95 per cent oxygen; 1 died on removal from the chamber and only 1 survived 72 hours residence.

These data emphasize the high degree of individual susceptibility to 95 per cent oxygen, and are in agree-

[2] A large series of phosgene-poisoned dogs treated with 60 per cent oxygen was studied by another group elsewhere and will be reported separately.

ment with the findings of others (19, 21). The general physiologic characteristics of oxygen poisoning noted in recent reviews by Bean (22) and Stadie (23) were observed in these experiments.

The lungs of oxygen-poisoned animals resemble liver in appearance and consistency, and drip fluid on section; the histologic picture resembles that of phosgene poisoning rather closely (24). Inflating such lungs brings out the emphysema, and grossly they then resemble phosgene-poisoned lungs rather than liver. In most instances the degree of lung damage was poorly correlated with clinical appearance and respiratory distress.

No control experiments were carried out with 40 per cent oxygen, as the series with 80 per cent oxygen, although small, showed no lethal effects. This latter series also served as a control for conditions of residence in the chamber.

RESULTS

The survival curves of the 3 series of experiments on dogs are shown in Figures 1, 2, and 3; the number of dogs in the control and experimental groups are shown in each figure. Attention is directed to the deaths occurring shortly after removal from the atmospheres of 95 and 80 per cent oxygen. These animals died with signs of acute anoxia in room air, whereas in the oxygen-enriched atmospheres they had not been cyanotic, despite dyspneic breathing. A detailed statistical analysis of the data makes the following statements probable:

1. According to Fisher's test for homogeneity (25) the 3 control series are comparable one with another and form a homogeneous set of data; therefore, they may be grouped together. This is true regardless of the time at which survival numbers are taken, *i.e.*, the percent survivals at 1, 2, 3, and 10 days are essentially the same in the 3 control groups. The number of delayed deaths in the controls for the 95 per cent series was somewhat unusual, but this does not alter the statistical homogeneity.

2. Comparison of the 95 per cent oxygen series with the *grouped* controls failed to show a very significant increase in survival at 24 hours ($P = 0.057$, Fisher's exact method) but did show a significant improvement at 2 days ($P = 0.03$) and 3 days ($P = 0.02$). The ultimate survival, based on that of the tenth day, was *significantly decreased* ($P = 0.02$) by treatment with 95 per cent oxygen when administered as described above.

3. Comparison of the 80 per cent oxygen series with the *grouped* controls showed that survival was not improved on a 1-, 2-, or 3-day basis, and that the ultimate, or 10-day, survival was significantly decreased ($P = 0.03$).

4. Comparison of the 40 per cent oxygen series with the *grouped* controls shows that this concen-

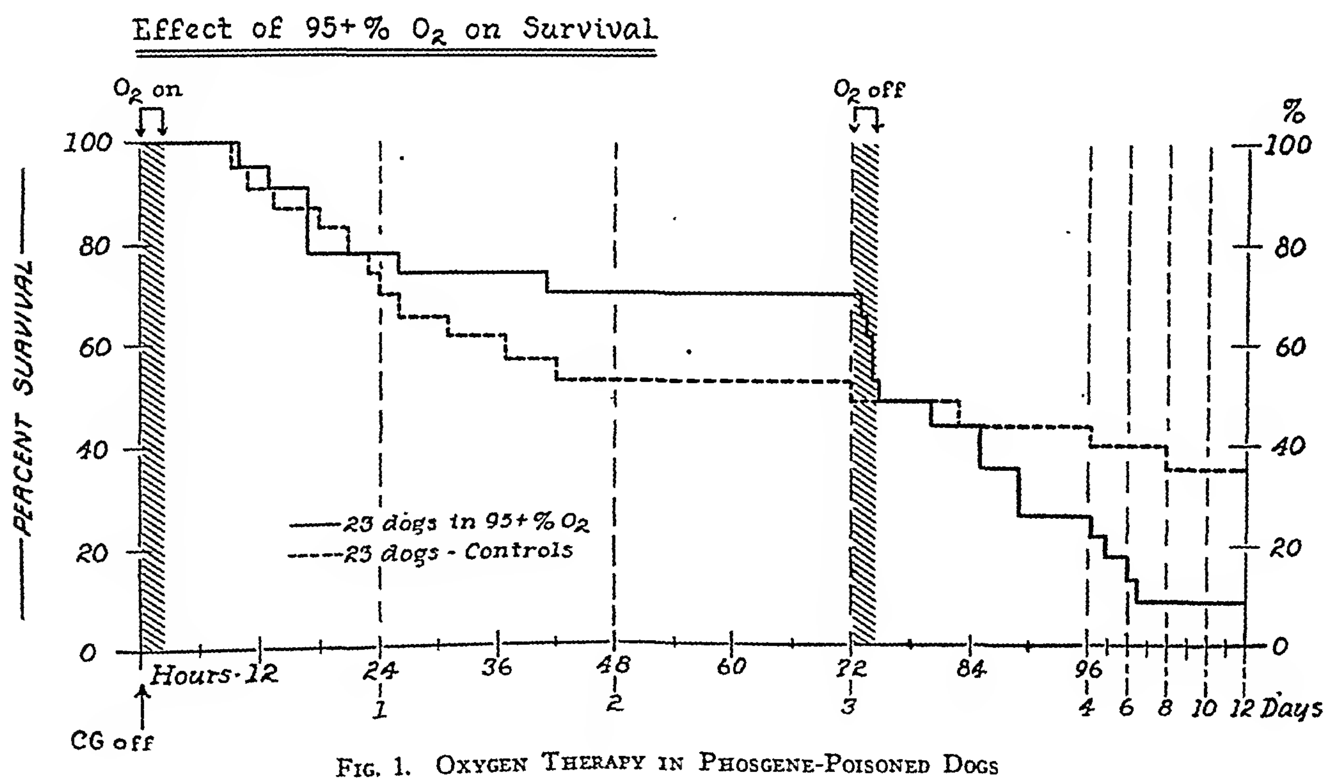

FIG. 1. OXYGEN THERAPY IN PHOSGENE-POISONED DOGS

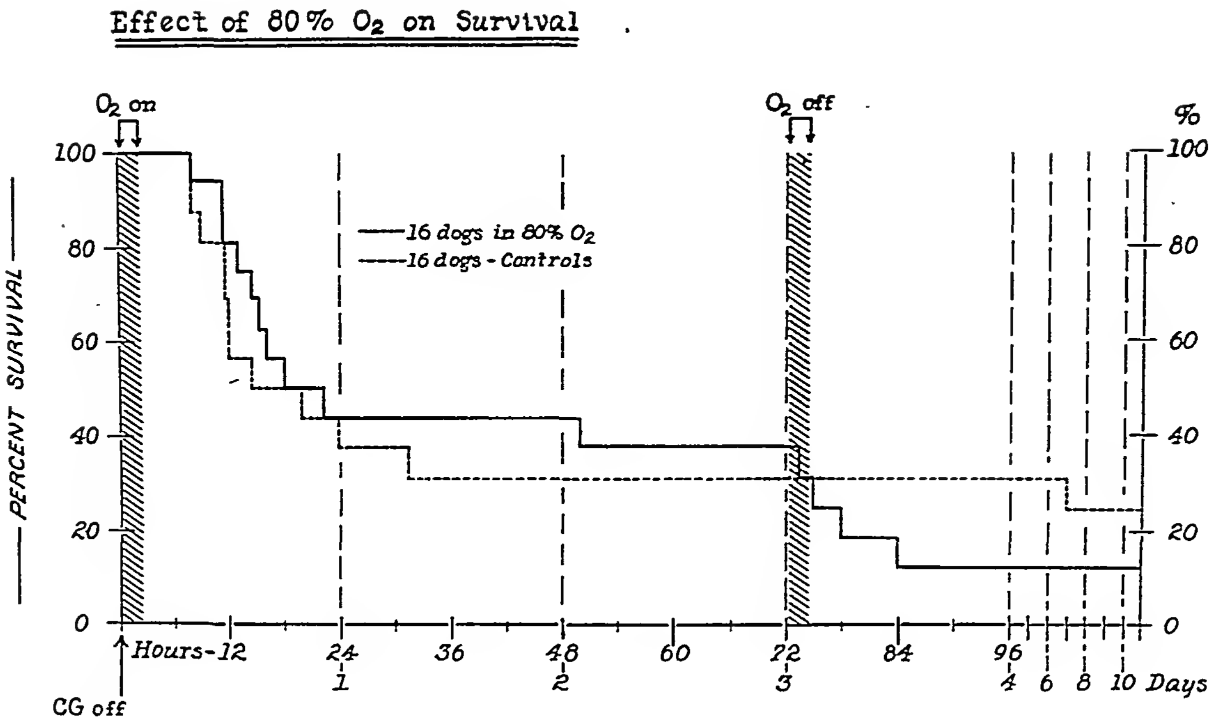

FIG. 2. OXYGEN THERAPY IN PHOSGENE-POISONED DOGS

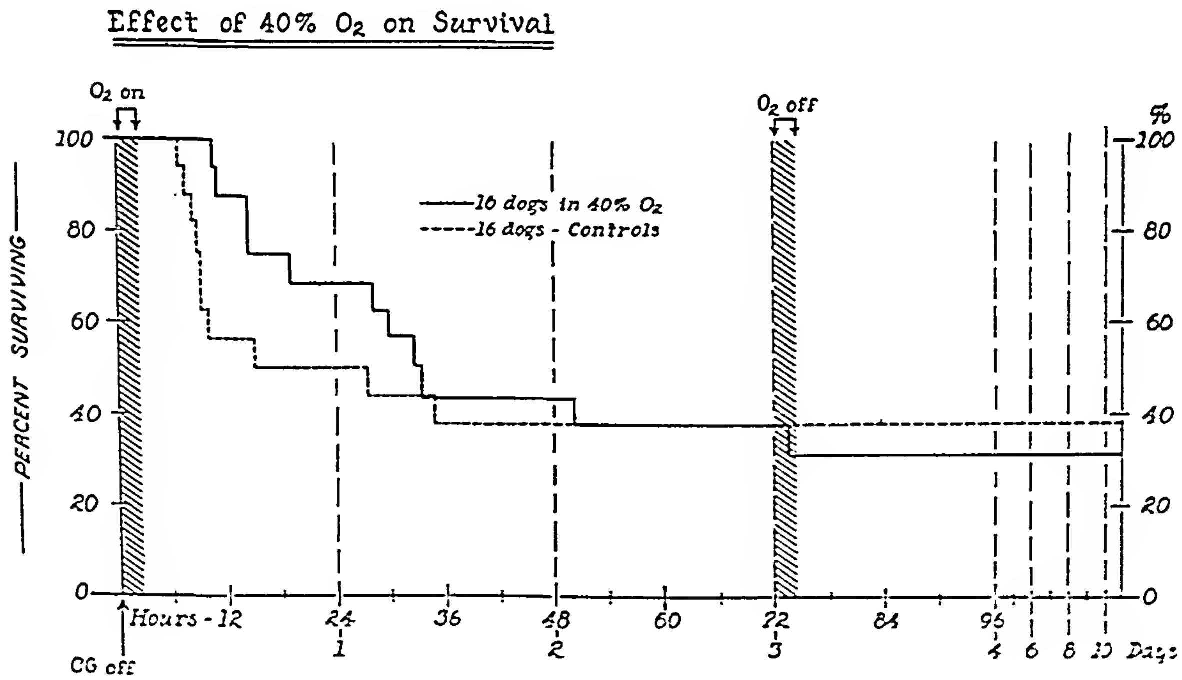

FIG. 3. OXYGEN THERAPY IN PHOSGENE-POISONED DOGS

tration of oxygen did not significantly influence either immediate or ultimate survival.

While the numbers of animals in each of the treated groups are comparatively small, the statistical analysis is supported by its agreement with the interpretations gained by inspection of the survival curves.

Judged by the responsiveness of the animals, their appetite, and the rate, depth, and ease of breathing, those treated with 40 per cent oxygen appeared to be in better clinical condition throughout than the controls, even up to the immediate premortem phase. In 80 per cent and 95 per cent oxygen this improvement was noted only during the first 36 to 48 hours; thereafter, the survivors appeared worse clinically than the controls living in room air. The surviving controls by this time gave clinical evidence of having passed the critical period.

No relation was found between the length of survival and the interval between gassing and beginning oxygen therapy. No effect of oxygen therapy was found on the rate of development of pulmonary edema as judged by the rate of hemoconcentration. The degree of edema at death (within 80 hours) was essentially the same for both groups, the treated animals having an average lung-to-body weight ratio of 4.1 and the control group an average of 4.2. The heart rate tended throughout to be slower in the oxygen-treated animals than in the controls. Bradycardia was found in normal animals residing in oxygen concentrations of 80 per cent or above.

At autopsy, gassed animals that had died in the 95 per cent oxygen, or within 1 or 2 hours after removal into room air, showed the liver-like lung of oxygen poisoning, but forced inflation of the lung restored the typical picture of phosgene poisoning. In the 80 per cent oxygen series, a few emphysematous blebs were present at death, while in the 40 per cent oxygen series the lungs were like those of typical phosgene poisoning; the latter was also true of the dogs of the 80 per cent and 95 per cent series which died 4 hours or longer after being returned to air.

The survival of 20 phosgene-poisoned rats was similarly unimproved by 95 per cent oxygen treatment. Their autopsy findings were like those described for dogs.

It was impossible to determine from either gross or histologic examination of the lungs and other tissues how oxygen therapy had influenced the course of phosgene poisoning, or whether oxygen poisoning had been superimposed on the phosgene poisoning. The pulmonary damage by the 2 agents is so similar as to defeat pathological methods of differentiation (24).

Five phosgene-poisoned rats were kept for 48 hours in 100 per cent oxygen maintained at ½ atmosphere, a condition which was proved not to produce oxygen poisoning in normal animals. The lungs of these rats were solid and liver-like; hence, the anatomic appearance of the lung after high oxygen therapy may be attributed to the absence of an inert gas and not to high oxygen tensions *per se*. This form of oxygen therapy also failed to lower the mortality rate.

Data on the mortality following residence in low oxygen tensions for 1 and 4 hours after gassing are shown in Table I. It is evident that this procedure did not increase the mortality of phosgene-poisoned rats, and comparison by the χ^2

TABLE I

Survival of phosgene-poisoned rats following residence in low oxygen atmospheres during the early phases of poisoning*

Dose of phosgene	Procedure	No. of rats	Hours in low oxygen	Average oxygen level	Rats surviving at: 24 hours	Rats surviving at: 72 hours
mgm.-min. per liter				*per cent*	*per cent*	*per cent*
2.32	Treated	5	1	10.6	60	60
	Treated	5	4	10.6	80	80
	Controls	10			20	20
2.90	Treated	5	1	10.7	60	40
	Treated	5	4	12.2	60	60
	Controls	10			50	50
2.38	Treated	5	1	10.8	60	20
	Treated	5	4	11.8	60	60
	Controls	10			70	70
1.61	Treated	10	4	10.8	80	70
	Controls	10			60	60
1.54	Treated	10	3	12.5	90	90
	Controls**	10			60	60
1.69	Treated	10	4	13.0	70	70
	Controls**	10			60	50

* CO_2 was 0.25 per cent or less; humidity and temperature equal to room air.

** Controls kept in similar 67-liter chamber with room air circulating.

test of the summed survivals of the rats treated for 4 hours, with their summed controls gives a P value of slightly less than 0.03, *i.e.,* a probably significant reduction of mortality. This, however, is not fully representative of the remaining 13 experiments, using 300 rats, in which oxygen percentage was varied between 10 and 18 per cent, CO_2 between 0.25 and 5 per cent, humidity between 35 and 100 per cent, and duration of treatment between 1 and 4 hours, beginning at once after gassing. In these latter experiments the data were ambiguous in the sense that consistent, repeatable results could not be obtained; some experiments gave decidedly favorable survival rates in the treated groups, whereas others gave equally unfavorable survival rates. In short, it appears that treatment with low oxygen tensions in this way was not deleterious; whether it was beneficial is uncertain. Variations in factors other than oxygen percentage appeared not to influence the results.

The rats in the low oxygen tensions experienced anoxia, as judged by the respiratory efforts, the color of their ears and retina, and their behavior; when they were removed from the chamber after 4 hours they had palpable rales. No animals remained in the low oxygen atmospheres longer than 4 hours, a time at which edema generally was about half maximal. The lung-to-body weight ratios of many of the rats which died a few hours after being returned to room air were low compared with the controls.

DISCUSSION

The data show that while continuous 95 per cent oxygen therapy delayed death after gassing, it failed to improve ultimate survival. In fact, 95 per cent oxygen therapy as administered to gassed dogs had an adverse effect on ultimate survival, due presumably to superimposed oxygen toxicity. This finding is not peculiar to the dog, according to the reports of Dumoulin and Charlier (18) and Soulie (19). It therefore assumes clinical importance, first because high concentrations of oxygen, administered for long periods, were required to banish cyanosis in phosgene casualties of World War I (1), and, second, modern techniques of oxygen therapy are capable of providing oxygen tensions which might lead to oxygen poisoning. Thus it would be unwise to administer oxygen tensions in excess of those necessary to saturate the hemoglobin. It is not reasonable to assume that unavoidable interruptions are a factor of safety in the so-called 100 per cent oxygen therapy by mask (26).

Although the data suggest that oxygen poisoning was superimposed on the phosgene poisoning, it is not possible to be certain of this at present. No differentiation could be made by either the histologic or physiologic methods used (24). Apparently, both induce similar if not identical pulmonary reactions and produce death by the same process, but oxygen at inhaled tensions of 600 to 760 mm. Hg requires a longer time factor. The wide variations in susceptibility to oxygen poisoning provide another important but unassessable consideration. A possible interpretation is that high tension oxygen therapy, by delaying death, permits accumulation of edema fluid in amounts greater than that which would have induced fatal anoxia in air; this extra fluid may then nullify therapy. The essential question must remain open for the present: Will high oxygen tensions administered to a lung damaged by phosgene give rise to processes which ultimately are just as lethal as the primary damage?

The data above suggest that oxygen therapy does little to prevent or break up the compound vicious cycle associated with the 2 forms of anoxia identified in phosgene poisoning: (*a*) an anoxic anoxia, dependent on the pulmonary edema, and (*b*) a stagnant anoxia arising from the shock-like hemodynamics connected with reduced blood volume and increased blood viscosity. Oxygen therapy alone may relieve the anoxic anoxia and improve cardiac action without materially improving the general capillary circulation. Yet attempts to improve the circulation by infusions during oxygen therapy have not been successful (27).

It does not follow that every case of phosgene poisoning presents this gloomy outlook. It is reasonable to anticipate that an unknown but probably small proportion of cases would die during the phase of maximal edema if not supported by oxygen therapy. This possibility should not be disregarded in the clinical management of the single case, statistics notwithstanding. On the other hand, the above data justify use of the alternate case method in assessing clinically the value of

oxygen therapy in the event of a mass exposure. The effects of sublethal anoxia on other tissues such as the kidney (28, 29), whose function is required during the subsequent restorative processes, indicate the use of oxygen. If it minimizes these later extra-pulmonary derangements, it is worthwhile.

While recognizing the mutual conflicts in the foregoing discussion, we nevertheless believe both points of view justified. However, it must be emphasized that a lessened ultimate mortality should be considered the only definitive criterion of benefit.

Drinker (20) recently proposed that anoxia of the pulmonary capillaries may cause, or exaggerate, edema initiated by other agents. This hypothesis might apply to phosgene poisoning, for blood oxygen analyses of gassed dogs show an anoxic and a stagnant anoxia present shortly after gassing (30) (Table II). This is also true of dogs poisoned by lower doses of phosgene (16). Generally, there is a partial recovery of normal saturation which is again reversed about the time edema becomes clinically detectable. It was noted that the more severe the immediate anoxemia, the sooner the animals died. Barcroft (31) reported similar changes in phosgene-poisoned goats.

TABLE II

Average and range of oxygen saturations of arterial and venous blood of dogs poisoned by an L(CT) 99 of phosgene

Oxygen saturation	Before gassing (8 exps.)	15 to 20 minutes after gassing (8 exps.)	2 to 5 hours after gassing (5 exps.)
		per cent	
Arterial oxygen saturation	96	82	80
	(94–99)	(58–93)	(68–90)
Venous oxygen saturation	62	21	26
	(37–91)	(11–84)	(16–47)

On the other hand, this hypothesis is unlikely to apply in phosgene poisoning for the following reasons: (*a*) Some gassed animals developed edema with typical findings without showing the early anoxemia (16, 27). (*b*) The dogs that died in the high oxygen atmospheres with 24 hours after exposure to phosgene showed full pulmonary edema. Oxygen toxicity does not occur within this time. (*c*) Exposure of phosgene-poisoned rats to low oxygen tensions during the period of development of edema did not increase the mortality over that of controls in air. (*d*) The bronchiolar obstruction of phosgene poisoning might cause anoxia of distal alveoli (24), the severity of which would depend on the oxygen tension of the pulmonary artery blood. These tensions have been found in many instances higher than the oxygen tensions used by Drinker and associates (20, 32) to increase lymphatic drainage from the lung, which is not in itself proof of pulmonary edema. The peribronchial tissues, which are supplied with arterial blood, are the first areas of the lung to show edema (24), and anatomically the lymphatics are more closely related to these tissues than to the alveolar areas (33). (*e*) Animals (34) and men (35) have been subjected to very low oxygen tensions without evidence of pulmonary edema, and the generally accepted view that anoxia of a capillary leads to increased permeability has recently been brought up for re-examination (36). Thus it seems unlikely that alveolar anoxia significantly influences the production of edema in phosgene poisoning.

The contradiction between clinical and experimental findings may be due to the fact that oxygen therapy in phosgene poisoning in man has never been subjected to as strict a test as that described above.

SUMMARY

1. Clinical opinion has assigned oxygen therapy a critical role in the treatment of phosgene poisoning, but this evaluation has not been confirmed in experimental studies previously reported.

2. One-hundred-ten dogs were exposed to an L(CT) 70 dose of phosgene. Half were then placed in a chamber operated dynamically, in oxygen atmospheres of 95 per cent, 80 per cent and 40 per cent, until death or for 72 hours. No improvement in ultimate survival was observed. Although death was delayed while the animals were in 95 per cent oxygen, ultimate survival was significantly decreased.

3. To assess the factor of oxygen poisoning in the above results, ungassed dogs were exposed to similar oxygen concentrations. Because the pulmonary damage from continuous exposure to high oxygen tensions was so similar to that of phos-

gene poisoning, it was uncertain whether the increased mortality in the 95 per cent series was due to superimposed oxygen poisoning.

4. Because of the possibility that the edema might have been related to pulmonary anoxia, gassed rats were kept in low oxygen atmospheres (10 to 18 per cent) for the first 1 to 4 hours after gassing. This treatment did not modify the course of the poisoning, nor did it decrease survival. In some experiments survival was significantly improved.

5. The experimental findings are discussed in relation to their clinical application.

BIBLIOGRAPHY

1. Chemical Warfare Medical Committee, Report No. 10. The administration of oxygen in irritant gas poisoning. H. M. stat. off., London, October, 1918.
2. Haldane, J. S., Lung irritant gas poisoning and its sequelae. J. Roy. Army Med. Cps., 1919, 33, 494.
3. Gilchrist, H. L., The Medical Department of the United States Army in the World War, Washington, Govt. print. off., 1926, Vol. 14. Chapter 7. Symptoms and treatment.
4. Laqueur, E., and Magnus, R., Über Kampfgasvergiftungen. V. Experimentelle und theoretische Grundlangen zur Therapie der Phosgenerkrankung. Ztschr. ges. exp. Med., 1921, 13, 200.
5. Sage, H. H., Acute phosgene poisoning; roentgen findings in the lungs; case report. Am. J. Roentgenol., 1944, 51, 9.
6. Shaw, R. E., A case of industrial phosgene gas poisoning. Univ. Leeds Med. Soc. Mag., 1941, 11, 147.
7. Steel, J. P., Phosgene poisoning; report on two cases. Lancet, 1942, 1, 316.
8. Carlisle, J. M., Pulmonary edema. J. A. M. A., 1943, 123, 947.
9. Jones, A. T., The treatment of casualties from lung irritant gases with particular reference to the use of oxygen and carbon dioxide mixture. J. Indust. Hyg. & Toxicol., 1940, 22, 235.
10. Gerber, I., Un cas d'intoxication mortelle par le phosgène. Rev. med. Suisse Rom., 1920, 40, 356.
11. Hegler, C., Ueber eine Massenvergiftung durch Phosgengas in Hamburg. I. Klinische Beobachtungen. Deutsche med. Wchnschr., 1928, 54, 1551.
12. Longcope, W. T., and Luetscher, J. A., Jr., Personal communication, 1944.
13. Chemical Warfare Medical Committee, Report No. 1. Notes on the pathology and treatment of the effects of pulmonary irritant gases. H. M. stat. off., London, 1918.
14. Chemical Warfare Medical Committee, Report No. 7. Changes observed in the heart and circulation and the general after-effects of irritant gas poisoning. H. M. stat. off., London, 1918.
15. Prentiss, A. M., Chemicals in War. McGraw-Hill Book Co.,New York and London, 1937.
16. Underhill, F. P., The Lethal War Gases. Yale University Press, New Haven, 1920.
17. Meek, W. J., and Eyster, J. A. E., Experiments on the pathological physiology of acute phosgene poisoning. Am. J. Physiol., 1920, 51, 303.
18. Dumoulin, E., and Charlier, R., Étude experimentale de l'oxygénothérapie dans l'intoxication par phosgène. Compt. rend. Soc. de Biol., 1938, 129, 500.
19. Soulie, P., Modifications expérimentales de la résistance individuelle de certains animaux a l'action toxique de l'oxygène. Compt. rend. Soc. de Biol., 1939, 130, 541.
20. Drinker, C. K., Pulmonary Edema and Inflammation. Harvard University Press, Cambridge, 1945.
21. Paine, J. R., Lynn, D., and Keys, A., Observations on the effects of the prolonged administration of high oxygen concentration to dogs. J. Thoracic Surg., 1941, 11, 151.
22. Bean, J. W., Effects of oxygen at increased pressure. Physiol. Rev., 1945, 25, 1.
23. Stadie, W. C., Riggs, B. C., and Haugaard, N., Oxygen poisoning. Am. J. Med. Sci., 1944, 207, 84.
24. Coman, D. R., Bruner, H. D., Horn, R. C., Jr., Friedman, M., Boche, R. D., McCarthy, M. D., Gibbon, M. H., and Schultz, J., Studies on experimental phosgene poisoning; I. The pathologic anatomy of phosgene poisoning, with special reference to the early and late phases. Am. J. Path., In Press.
25. Fisher, R. A., Statistical Methods for Research Workers. Ed. 7, Oliver and Boyd, London, 1938, p. 88–97.
26. Comroe, J. H., Jr., Dripps, R. D., Dumke, P. R., and Deming, M., Oxygen toxicity: the effect of inhalation of high concentrations of oxygen for 24 hours on normal men at sea level and at a simulated altitude of 18,000 feet. J. A. M. A., 1945, 128, 710.
27. Unpublished data.
28. Phillips, R. A., Dole, V. P., Hamilton, P. B., Emerson, K., Jr., Archibald, R. M., and Van Slyke, D. D., Effects of acute hemorrhagic and traumatic shock on renal function of dogs. Am. J. Physiol., 1946, 145, 314.
29. Shorr, E., Zweifach, B. W., and Furchgott, R. F., On the occurrence, sites and modes of origin and destruction, of principles affecting the compensatory vascular mechanisms in experimental shock. Science, 1945, 102, 489.
30. Patt, H. M., Tobias, J. M., Swift, M. N., Postel, S., and Gerard, R. W., Hemodynamics in pulmonary irritant poisoning. Am. J. Physiol., 1946, 147, 329.

31. Barcroft, J., Anoxemia as a factor in acute gas poisoning. J. Roy. Army Med. Cps., 1921, 36, 1.
32. Warren, M. F., Peterson, D. K., and Drinker, C. K., The effects of heightened negative pressure in the chest, together with further experiments upon anoxia in increasing the flow of lung lymph. Am. J. Physiol., 1942, 137, 641.
33. Miller, W. S., The Lung. Charles C. Thomas, Springfield, Ill., 1937.
34. Van Liere, E. J., Anoxia, Its Effect on the Body. The University of Chicago Press, Chicago, 1942.
35. Gibbs, F. A., Gibbs, E. L., Lennox, W. G., and Nims, L. F., The value of carbon dioxide in counteracting the effects of low oxygen. J. Aviation Med., 1943, 14, 250.
36. Henry, J. P., Klain, I., Movitt, E., and Meehan, J. P., The effects of anoxia on the capillary permeability of the human arm. Fed. Proc., 1946, 5, 44.

THE EFFECTS OF CONTINUOUS PRESSURE BREATHING ON KIDNEY FUNCTION [1]

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Continuous pressure breathing is a technique developed to increase the alveolar oxygen tension by breathing oxygen at higher than ambient pressure. In other words the pressure within the alveoli of the lungs is higher than that outside the chest wall. It is found that as much as 16 mm. Hg of such extra pressure can be tolerated for periods of 30 to 60 minutes by the average healthy man. However, as the extra pressure is increased above this figure, evidence of a deleterious effect upon the circulation becomes increasingly apparent. At pressures of 25 to 40 mm. Hg circulatory collapse and fainting become frequent. Often in cases in which this does not ensue there may develop sensations of impending collapse with pallor and sweating, necessitating termination of the test. Although the blood pressure remains above the control level the pulse is small and difficult to feel and the cardiac output is diminished (1). When using these higher pressures not only is much blood pooled in the veins of the arms and legs but also there is a progressive hemoconcentration due to the passage of fluid from the blood stream into the tissue spaces under the influence of the raised intravascular pressure (2). A considerable fraction of the total blood volume is thus more or less sequestrated from the general circulation under these conditions and when collapse ensues it is in large part due to decreased effective blood volume. The actual total volume of blood thus isolated cannot be readily assessed and there is need of a reliable but simple test which could be used to assay the impairment of circulation induced by pressure breathing.

With this aim in view we studied the blood pressure changes occurring during continuous pressure breathing. This was determined in the usual manner on the arm. Such an approach proved unsatisfactory since a fall in the pressure was usually only observed when the subject was on the verge of syncope. Otherwise the blood pressure was the same as the control value or, as was usual, it was somewhat elevated. We then decided to investigate the value of a determination of the changes in kidney function produced by pressure breathing.

METHOD

The specific function of the kidney studied was its capacity to excrete urea. This information is obtained by determining the rate of urea excretion during any period and dividing this by the blood urea concentration at the time, thus obtaining a value essentially the same as the kidney clearance. Urea was chosen because of the accuracy with which it can be determined and the constancy of its clearance in a given individual under standard conditions. In addition it has been shown to be responsive to physiological changes in the circulatory system which take place in such conditions as severe exercise (3) and hemorrhage (4). The test was carried out as follows: The subject went without breakfast in the morning and drank a liter of water on rising and 500 ml. per hour thereafter until the completion of the test. Within 2 hours a good diuresis usually developed and the determination of function was then started. The subject emptied his bladder and noted the exact time. Thirty minutes later a blood sample was taken and 30 minutes after that, the first urine collection was made. Immediately after completion of this 1-hour control period the subject pressure-breathed for 30 minutes.

Either compressed air or pure oxygen was used and applied by an Army A-13 pressure-breathing mask. Pressures were measured at the mask and determined by observing a manometer connected to a tube introduced into the mask. In order to ensure a full application of the pressure the subjects were instructed not to restrict the passage of gas to the lungs by closure of the glottis. No counter-pressure was applied to the body. A blood sample was taken midway through the pressure-breathing period and the urine collected at the end. Observations were continued during the post-pressure-breathing period for 2 or 3 consecutive hourly periods. Thus each test involved a series of 4 or 5 periods which together occupied a whole morning.

Four subjects were studied with repeated tests. On any given test the breathing pressure remained constant throughout the pressure-breathing period. Duplicate run-

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[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Southern California.

TABLE I

Representative table showing the decrease in kidney function during a 30-minute period of breathing at 40 mm. Hg super added pressure

The equation below illustrates the method of calculating the reduction in kidney function produced by the pressure breathing and the figures so obtained are those depicted in Figures 1, 2, 3, and 4. In addition the table illustrates the lag in return of function after pressure breathing is stopped.

June 10, 1946
Subject: E. K.
Pressure: 40 mm. Hg

Period	Time	Urine urea	Blood urea	Urea clearance	Kidney function (control)
.	*min.*	*mgm. per hr.*	*mgm. per 100 ml.*	*ml. per hr.*	*per cent*
1	68	906	19.8	4580	100.0
2 P.B. Period	32	426	18.3	2330	50.9
3	31	106	18.6	570	12.4
4	47	847	18.9	4480	97.8

$$\frac{\text{Urea clearance Per. 2}}{\text{Urea clearance Per. 1}} = \frac{23.3}{45.8} = 50.9 \text{ per cent.}$$

were carried out on all subjects using 10, 20, 30, and 40 mm. Hg mask pressure.

The urea in the blood and urine was determined by the urease technique of Addis (5). The rate of excretion of urea was calculated on a per hour basis for each period (pre-pressure-breathing, pressure-breathing and the two post-pressure-breathing periods). This hourly rate was divided by the urea concentration of mgm. per cent found in the blood taken at the middle of the period. (Determinations of the blood urea showed no significant change throughout the morning of a given test.) The quotient so obtained was taken as the measure of kidney function during any particular period. The value for the kidney function during the pre-pressure-breathing period of a given test was taken as 100 per cent and the kidney functions found during the succeeding periods were calculated on this basis.

RESULTS

It is apparent from Table I that the pressure breathing has caused a decrease in kidney function not only during the period of active pressure breathing (Period 2) but also during the post-pressure-breathing period (Period 3). In Figure 1 are shown the results of a series of such tests on the same subject, each circle representing the reduction in kidney function during the pressure-breathing period of one test. Figures 2, 3, and 4 represent similar series of tests for 3 other subjects. It will be seen that at 40 mm. Hg pressure the function was reduced to between 20 to 50 per cent of normal.

A pressure of 10 mm. Hg shows practically no effect; but, beginning with 20 mm. Hg there is a definite functional depression which becomes greater with successively higher pressures. In many experiments the function remains depressed for an hour or more after the resumption of normal breathing. Figure 5 illustrates this point. The continuation of the depression of urine volume and urea clearance during the first post-pressure-breathing period is marked. It may be noted that when the urine volume decreases to levels of 0.1 ml. per min., as it did in the post-pressure-breathing

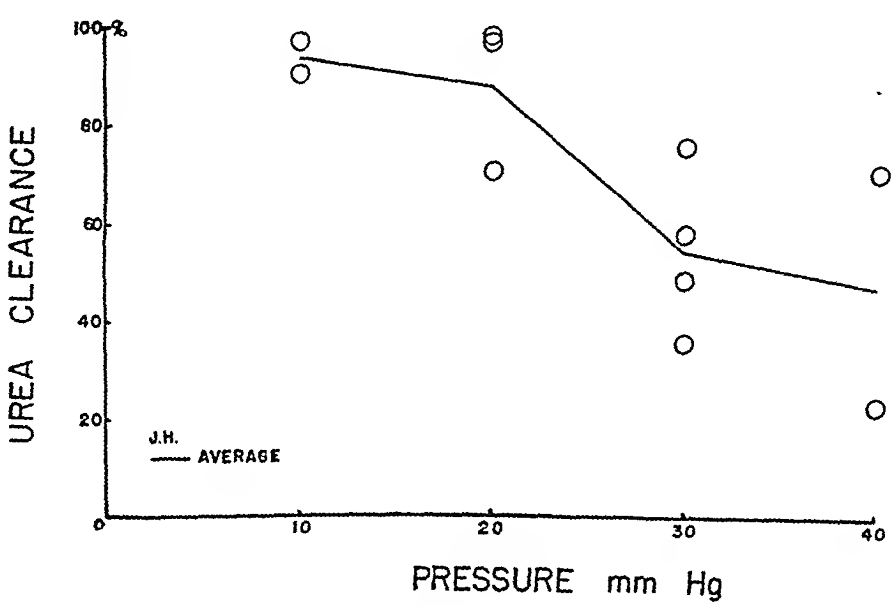

FIG. 1

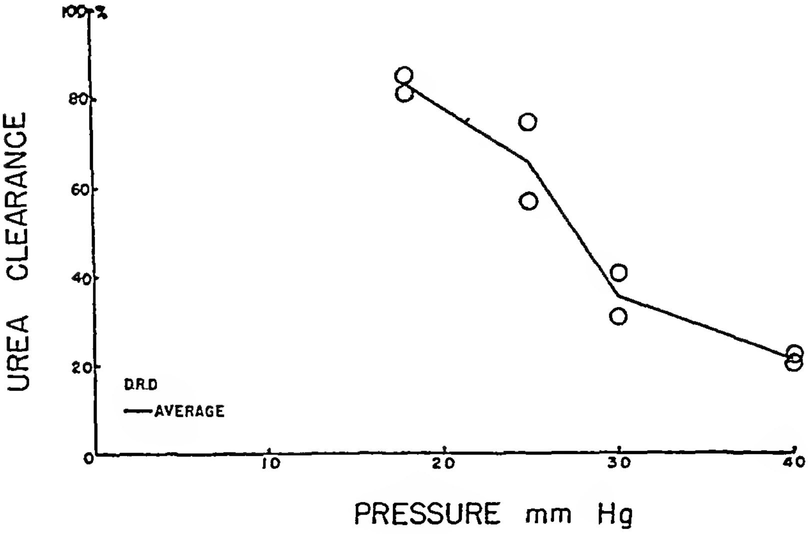

FIG. 2

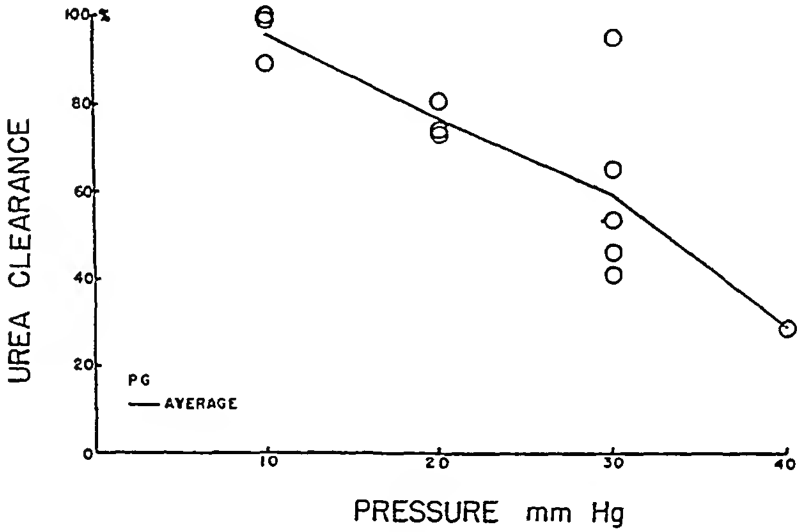

FIG. 3

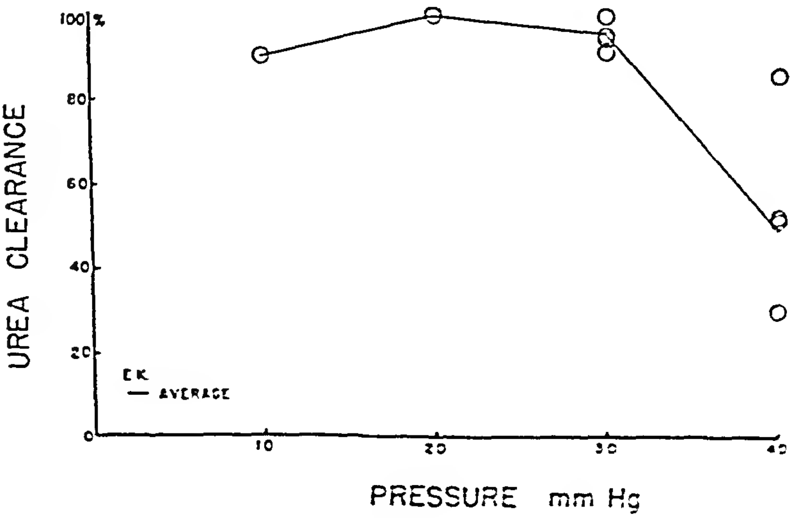

FIG. 4

period represented in this case, the urea clearance determination becomes less accurate (6). Figure 6 illustrates a case in which the function during the post-pressure-breathing period is markedly diminished below that during the pressure-breathing period. However it must be remembered that the charts give only the average of function during each period. It is not possible to determine the

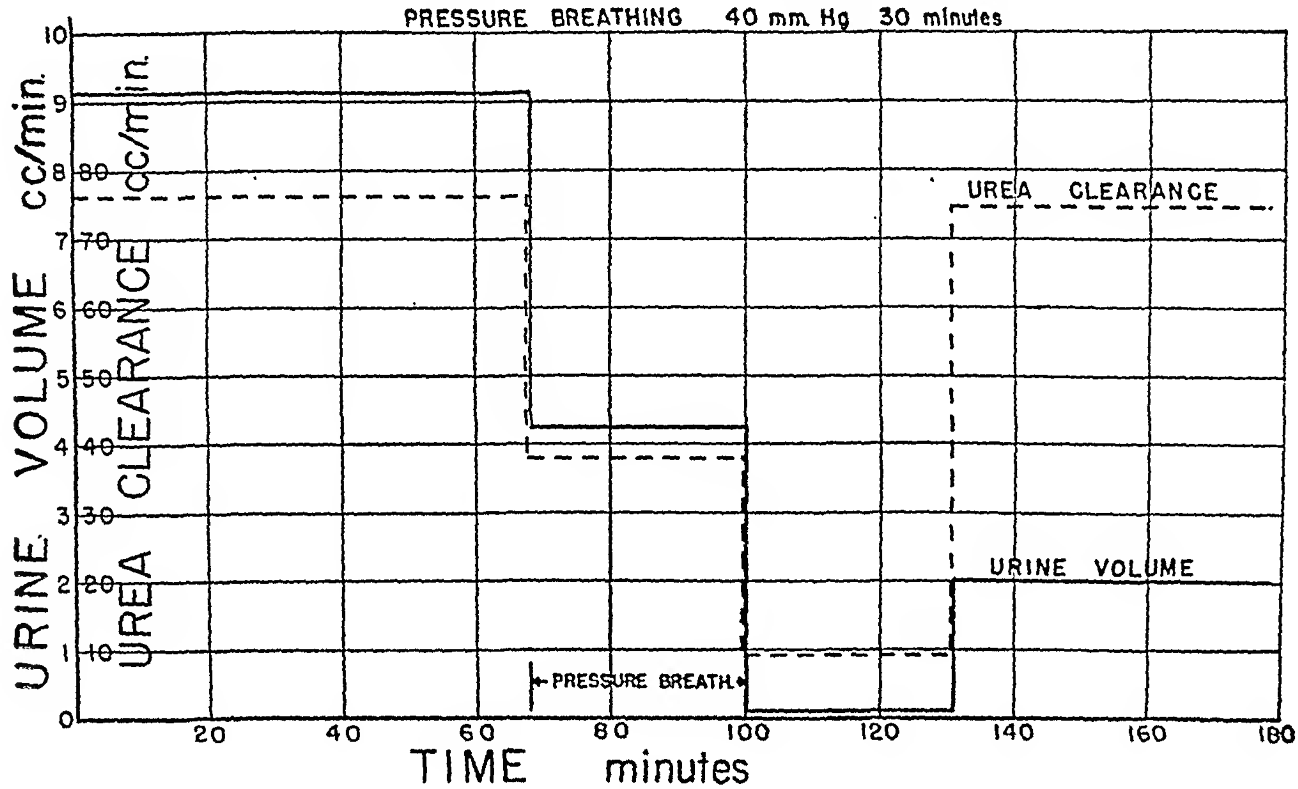

Fig. 5

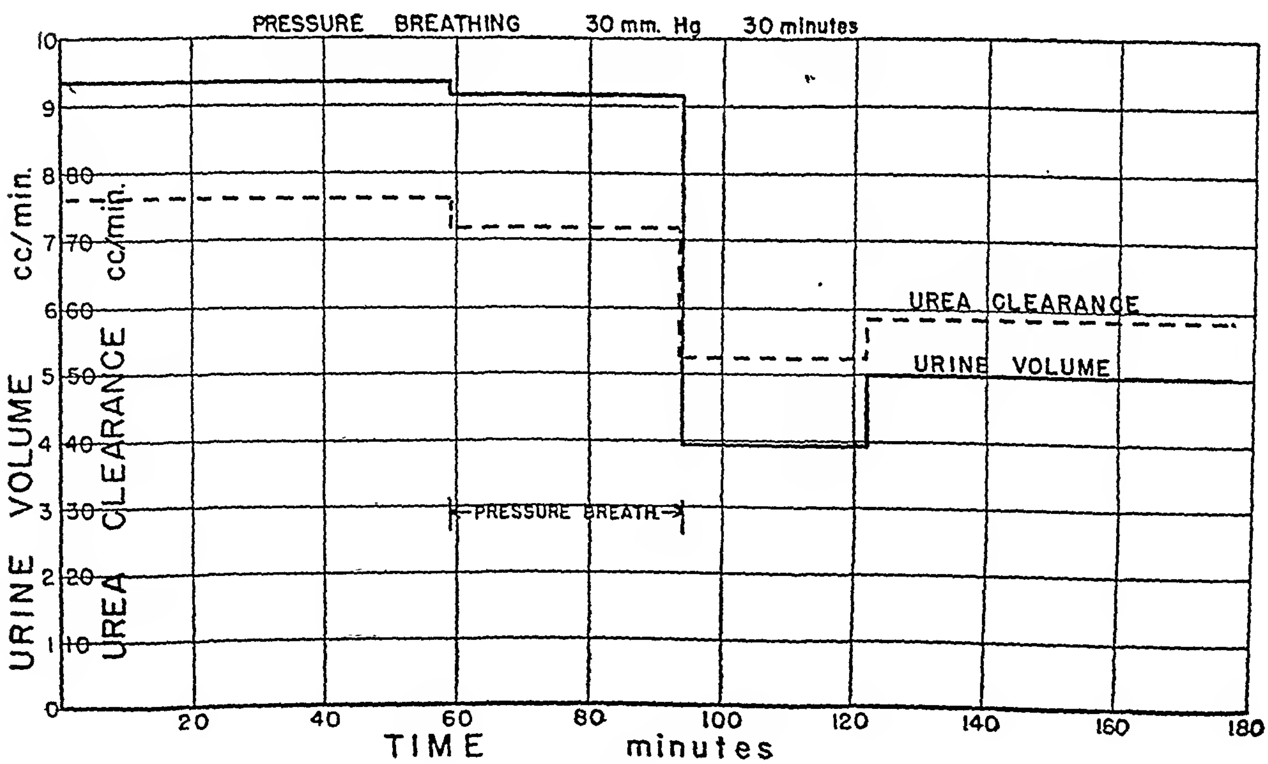

Fig. 6

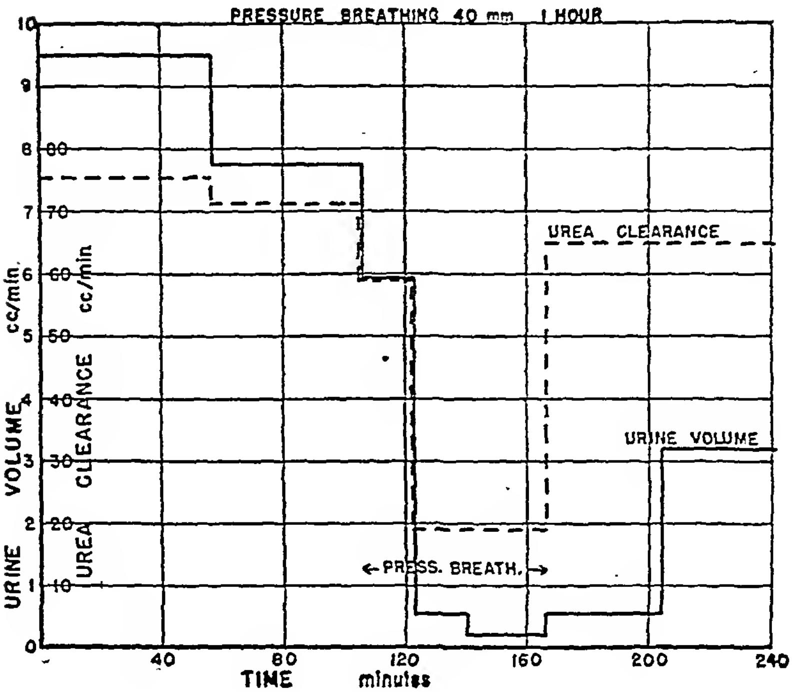

Fig. 7

function for each minute. Therefore, for the case shown in Figure 6, it does not follow that the function during the last minute of the pressure-breathing period was actually lower than that of the first minute of the after period.

This prolonged depression in function following the pressure breathing suggested that there is a certain "inertia" of function prolonging response beyond the causal period. The question arose whether there was a corresponding delay in the decrease in function at the onset of the pressure-breathing period. To test this, an experiment was carried out in which the subject continued pressure-breathing for 1 hour (Figure 7). This period was subdivided into 3 periods in each of which a separate function determination was carried out. It is apparent that marked depression does not occur immediately and that during the first 15 to 20 minutes the function remains relatively high.

DISCUSSION

Reference may be made to some of the work that has been published in recent years indicating that the kidney circulation is early reduced in conditions that embarrass the general circulation (7 to 9). The results of these workers in addition to that of others support the view that a measurement of kidney function can be used to determine the degree of circulatory stress. Our findings are definitely in line with this. Care must be taken in every particular case to evaluate the action of additional factors, such as kidney disease and specific renal toxins, which can affect kidney function. Pain also has been shown to diminish renal function (10). The exact mechanism of its action on the kidney has not yet been decided. There was some degree of discomfort in breathing at the higher pressures and this action may have contributed to the reduction in kidney function seen here.

Pressure breathing may be considered a useful experimental method by which definite circulatory stress can be produced rapidly and with relative safety. We feel that the degree of depression of kidney function that we obtained is proportional to the magnitude of the circulatory stress produced. Used in this way the procedure has been of some value to us in determining the efficacy of supportive measures which may be used to combat the deleterious effect of pressure breathing on the circulation.

Our results indicate that pressure breathing of moderate magnitude can be tolerated by normal healthy individuals for appreciable times, *i.e.*, ½ to 1 hour. The higher pressures cannot be tolerated

so long. We believe that pressure breathing might be used as a test of circulatory fitness. It would seem probable that individuals with reduced circulatory reserve such as is found in persons employed in sedentary occupations would be affected by those lower pressures which do not have very much effect on physically active people in good circulatory condition. The degree of effect on the circulation could, we believe, be readily estimated by determining the effect on kidney function. Not only does such a test give an opportunity for quantitative measurements but it can be carried out without producing the alarming and uncomfortable symptoms associated with circulatory collapse.

In conditions in which the amount of actively circulating blood is reduced by loss of fluid or pooling in inactive areas the vasomotor system will cut down on blood flow through regions that can tolerate this temporarily. The circulation through the kidney behaves somewhat like that through the skin in this respect and kidney blood flow can be reduced to $1/10$ to $1/20$ of normal resting volumes when strain is imposed on the circulatory system (11). The kidney circulation can in fact serve as an important reserve for the general vascular system. Not only is a certain volume of blood made directly available by the reduction in kidney volume following renal vaso-constriction but in addition it may be noted that normally $1/4$ of the blood in the great veins, heart, lungs and aorta is concerned with the renal circulation and that when blood flow through the kidney is reduced this blood can then be utilized to serve the vital areas of the body.

The question might be raised as to the "wisdom of the body" in sacrificing kidney function in conditions putting a strain on the circulatory system. If this strain is not prolonged beyond a few hours then cutting down of the blood flow through the kidney would seem to be a proper mechanism to help tide the circulatory system over a temporary embarrassment. The concomitant temporary reduction in kidney function would not be harmful. Thus, a normal individual can remain for at least a day without kidney function without showing otherwise any serious disturbance. Upon the resumption of activity this organ can quickly make up for the period of inactivity. We might speak of the body going into a "renal debt" in order to help the circulatory system carry on during a period of temporary stress. Naturally there should not be complete cessation of renal blood flow for any appreciable duration since this may lead to irreparable kidney damage if lasting more than 1 hour (12).

However, such a mechanism is not without its dangers. If the circulation cannot be established within a reasonable time the continued impairment of kidney function would lead to serious consequences. This can be the case in prolonged traumatic shock and infectious diseases causing peripheral circulatory failure (7). It is probable that continued dehydration can also be dangerous for this reason. Finally if renal function had already been inadequate for a prolonged period before the imposition of circulatory stress then a further reduction in renal blood flow even for a few hours could be a serious matter.

The lag in the return of kidney function to normal after cessation of pressure breathing is of interest. It represents to some extent the time required for the circulatory system to return to normal. However, this period is normally very brief since the blood pooled in the limbs is immediately available for active circulation on the cessation of pressure breathing. Some of the delay is due to the time (10 to 20 minutes), needed for return to the blood of fluid that had escaped into the extravascular compartment. In addition, time is needed for the kidney tissue to reestablish itself after proper blood flow has been started again. Thus Selkurt (13) has shown that after clamping the renal arteries for 20 minutes the return of kidney function to normal was delayed at least 2 to 3 hours after release of the artery, whereas direct measurement of blood flow showed a much more rapid restitution.

An additional important mechanism, however, which may account for much of the prolonged lag lies in the possibility of hormonal activity. The urine volume changes during the test suggest that an antidiuretic (presumably posterior pituitary) action may enter into the picture. Since pituitrin depresses urea clearance in addition to the urine volume, posterior pituitary activity may also play a significant part in the lag in return of kidney function to normal after cessation of pressure breathing (3). That this hormone is involved is suggested by the report of Brun *et al* (14) who studied kidney function during the circulatory collapse pro-

duced by prolonged maintenance of the upright position on the tilt table. They found that the inulin and diodrast clearances were reduced during the period of collapse and for periods of a quarter of an hour or more thereafter. The subjects drank large amounts of water so that they were well hydrated and were excreting large volumes of urine prior to the collapse. Following collapse the urine volume was markedly diminished and remained low for periods of 1 hour or more. They present evidence to support the view that this reduction in urine volume is due to increased secretion of an antidiuretic hormone. Our results are similar with respect to changes in urine volume. It should be noted that the urine volumes in our cases were depressed for a considerable period after the pressure breathing and that the urea concentration in the urine was increased at this time, although the clearance was depressed. Our findings differ from those of the above mentioned workers to the extent that we did not have symptomatic circulatory collapse and that the blood pressure was well maintained, whereas their subjects showed very low blood pressures during the period of reduced kidney function.

SUMMARY

1. Four subjects submitted to 30-minute periods of continuous pressure breathing at 10, 20, 30 and 40 mm. Hg above ambient pressure showed a progressive reduction of kidney function which, with the higher pressures, reached approximately 20 to 50 per cent of normal values.

2. This depression in function persisted for 1 to 2 hours after the cessation of the pressure-breathing period. The possible mechanism of this continued depression is discussed.

3. These results support the view that estimations of kidney function may be used as a measure of the circulatory stress induced by a reduction of effective blood volume.

4. It is suggested that pressure breathing should be investigated as a test of circulatory fitness by employing estimations of disturbance of kidney function as an index of the extent of its effect upon the circulatory system.

BIBLIOGRAPHY

1. Cournand, Andre. Personal communication.
2. Henry, J. P., and Meehan, J., Estimations of the decrease in effective blood volume when pressure breathing, to be published.
3. Addis, T., and Drury, D. R., The rate of urea excretion; effect of various other factors than blood urea concentration on rate of urea excretion. J. Biol. Chem., 1923, 55, 629.
4. Selkurt, E. E., Renal blood flow and renal clearance during hemorrhagic shock. Am. J. Physiol., 1946, 145, 699.
5. Addis, T., Urea determinations in blood and urine. J. Lab. & Clin. Med., 1925, 10, 402.
6. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Williams and Wilkins, Baltimore, 1931, Vol. 1, p. 345.
7. Maegraith, B. G., Havard, R. E., and Parsons, D. S., Renal syndrome of wide distribution induced possibly by renal anoxia. Lancet, 1945, 2, 293.
8. Lauson, H. D., Bradley, S. E., and Cournand, A., The renal circulation in shock. J. Clin. Invest., 1944, 23, 381.
9. Phillips, R. A., Dole, V. P., Hamilton, P. B., Emerson, Kendall., Archibald, R. M., and Van Slyke, D. D., Effects of acute hemorrhagic and traumatic shock on renal function of dogs. Am. J. Physiol., 1946, 145, 314.
10. Wolf, G. A., The effect of pain on renal function. A. Research Nerv. & Ment. Dis., Proc., 1943, 23, 358.
11. Cournand, A., *et al.*, Studies of the circulation in clinical shock. Surgery, 1943, 13, 964
12. Scarff, R. W., and Keele, C. A., The effect of temporary occlusion of the renal circulation in the rabbit. Br. J. Exper. Path., 1943, 24, 147.
13. Selkurt, E. E., Comparison of renal clearances with direct renal blood flow under control conditions and following renal ischemia. Am. J. Physiol., 1946, 145, 376.
14. Brun, C., Knudsen, E. O. E., and Raaschou, F., Kidney function and circulatory collapse. J. Clin. Invest., 1946, 25, 568.

LIVER BLOOD FLOW IN PREGNANCY—HEPATIC VEIN CATHETERIZATION [1]

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Numerous liver function tests have been devised and most investigators agree that some disturbance in liver function occurs during the toxemias of pregnancy. These changes are of relatively small magnitude and the clinical use of these tests in pregnancy is not widespread. The technique of venous catheterization with resultant sampling of blood directly leaving the liver offers further opportunities for study of liver function.

METHODS

A method of estimating hepatic blood flow in man based on the "Fick" principle has been described by Bradley and coworkers (1). In their article they point out that "The 'Fick' principle may be applied to any organ provided three facts are known: (*1*) the concentration of some substance, *X*, in the blood entering the organ, (*2*) the concentration of *X* in the mixed venous blood leaving the organ, and (*3*) the total amount of *X* removed from the blood by the organ each minute. Given these data, the blood flow through the organ per minute may be calculated by dividing the total removal rate of *X* by the amount of *X* removed from each milliliter of blood as it traverses the organ."

The removal rate of *X* by the liver cannot be measured directly, but an indirect method is available if *X* can be given at such a rate that its blood level remains constant. Under these conditions, the infusion rate equals the hepatic removal rate, provided that the extraction of *X* depends entirely upon hepatic activity. Bromsulfalein (BSP) was used as the test substance since it apparently satisfied these conditions.

The calculation of hepatic blood flow then can be determined by the following formula:

$$EHBF = \frac{R}{0.01\,(P - H)} \times \frac{1}{1 - \text{Hematocrit}}$$

Where $EHBF$ = estimated hepatic blood flow,
R = removal rate or the infusion rate in mgm. per minute,
P = peripheral venous concentration of BSP in mgm. per minute,
H = hepatic venous concentration of BSP in mgm. per minute.

Hematocrit was from anticoagulated blood drawn from the hepatic vein in most cases. Four hematocrits were always taken and the average of the 4 used in the calculations. Occasionally peripheral vein blood would be used for hematocrit determination because of temporary difficulty in obtaining enough hepatic vein blood through the catheter: in Tables I, II, and III these peripheral vein hematocrits are starred (*).

If the concentration of BSP in the peripheral blood is changing, hepatic blood flow may also be estimated if the plasma volume is determined. In this case, R (the removal rate of BSP by the liver) $= I$ (the infusion rate mgm. per minute) plus or minus $\Delta P \times V$ where $\Delta P =$ the rate of change in concentration of BSP in the peripheral blood in mgm. per ml. per minute and $V =$ plasma volume in ml. Plus or minus depends on whether the concentration of BSP in the peripheral blood is rising or falling. If the peripheral concentration is rising, $R = I - (\Delta P \times V)$ since less dye is being removed than is administered. If the peripheral concentration is falling, $R = I + (\Delta P \times V)$ for the converse reason.

Samples of hepatic vein blood are obtained with more or less ease by catheterization of the right hepatic vein with a 100-cm. No. 8 ureteral catheter with a slightly curved through-and-through tip with one eye. The catheter, through which an isotonic saline infusion is running, is inserted in the median basilic vein under local anesthesia. With the patient in the supine position on a fluoroscope table, the catheter is then passed into the subclavian vein, the superior vena cava, the right atrium, the inferior vena cava, and the right hepatic vein. The patient experiences no discomfort during this procedure. The most difficult part of the procedure is to get the tip of the catheter past the right atrium and into the inferior vena cava rather than down into the right auricle and ventricle. In advanced pregnancies this was more difficult than in the non-pregnant cases presumably because of increased angulation of the vena cava at this point due to elevation and deviation of the heart to the left by the upward displacement of the diaphragm and liver. Apprehensive patients with small peripheral veins were occasional failures

[1] This study was carried out with the aid of a grant from the Commonwealth Fund.

TABLE I

The average EHBF in non-pregnant women with no evidence of liver disease

Subject	Age in years	Diagnosis	Surface area	Average serum concentration BSP: P	Average serum concentration BSP: H	Average extraction percentage $\frac{P-H}{P}$	Total removal rate BSP	Hepatic blood flow	Hepatic blood flow	Average ΔP	Plasma volume	Hematocrit	Blood volume	EHBF per cent of total blood volume
			*M.*²	*mgm. per cent*		*per cent*	*mgm. per min.*	*ml. per min.*	*ml. per min. per 1.73 M.*²					
R. S.	26	Salpingitis	1.45	.81	.28	69.1	3.92	1402	1674	.0000656	2270	39.60*	3760	37.2
R. L.	23	Salpingitis	1.69	1.03	.58	43.10	4.86	1737	1779	.0000844	2688	36.00	4200	41.3
H. M.	28	No disease	1.68	1.87	.98	45.80	6.79	1270	1307	.0002323	2688	35.70	4180	30.4
R. C.	22	Salpingitis	1.43	2.18	1.30	39.80	6.95	1410	1706	.0001236	2315	37.10	3680	38.3
F. G.	22	Salpingitis	1.58	3.80	3.23	13.95	6.59	1736	1900	.0004564	2435	38.85	4050	42.8
F. J.	32	Salpingitis	1.75	3.53	2.43	28.50	7.65	1192	1177	.0000788	2582	38.80	4220	28.2
E. S.	25	Gonococcal cervicitis	1.52	2.42	1.60	32.80	5.98	1385	1576	.0003530	2315	41.40	3950	35.0
G. P.	29	Post-abortal salpingitis	1.56	1.17	.45	59.50	5.30	1297	1438	.0008445	2635	34.50	4020	32.2
M. F.	33	Psycho-neurosis	1.50	2.74	1.94	30.40	6.30	1313	1515	.0001425	2336	40.10	3900	33.6
P. T.	22	Salpingitis	1.52	1.81	.94	48.10	5.93	1203	1370	.0001000	2380	39.80	3950	30.4
M. L.	35	Bartholin cyst	1.44	1.22	.45	62.10	5.94	1298	1560	.0002770	2360	36.50	3720	34.8
M. L.	25	4 weeks postpartum	1.55	2.27	1.53	32.30	6.20	1264	1410	.0002150	2850	28.90	4000	31.6
L. S.	32	Salpingitis	1.38	1.64	.84	47.40	6.20	1223	1535	.0000534	2400	31.25	3490	35.0
M. W.	21	Salpingitis	1.58	2.74	1.99	26.20	6.76	1547	1695	.0001430	2780	31.40	4050	38.1
M. C.	22	25 days postpartum	1.67	2.21	1.20	45.40	7.68	1517	1573	.0007145	2700	35.50*	4190	36.2
Average									1548					35

* Indicates peripheral vein hematocrit. All others from hepatic vein. (See page 952.)

when peripheral venous spasm rendered the catheter immovable except for withdrawal. Not leaving the catheter in the body for longer than 90 minutes was preferred after 2 patients developed peripheral phlebitis when the catheter had been left in the veins for longer than 2 hours; the phlebitis disappeared rapidly under simple treatment.

The BSP infusion was allowed to run into the same arm through which the catheter was inserted and samples of peripheral blood were withdrawn from the opposite arm. The concentration of BSP in the peripheral blood was considered equavalent to afferent blood to the liver since the liver presumably is the exclusive site of removal of the dye.

In pregnancy it would be necessary to take into account the action of the fetal liver in removing BSP were it not for the fact that bromsulfalein does not cross the placenta. Two patients in the second stage of labor were given bromsulfalein intravenously in quantity sufficient to keep the peripheral concentration high for a period of 20 to 30 minutes prior to delivery of the baby. Immediately after delivery, samples of the blood from the umbilical cord showed no BSP present whereas the mother's peripheral blood concentration was still very high.

For a complete discussion of the method of determining the BSP concentration, of the validity of the method, and of the calculation of the *EHBF*, the reader is referred to Bradley *et al* (1). Our method of BSP determination was essentially the same as theirs. However, we did work with higher blood levels of BSP (*i.e.*, 1.0 mgm. per cent or over for the hepatic blood levels, since these are always lower than the peripheral) because below this level we found an increasing percentage of error in the plasma BSP recoveries. If the plasma concentration was 1.0 mgm. per cent or over, the percentage error was only 2½ to 5 per cent. Since the best plasma recoveries usually showed a 5 per cent loss, all plasma levels were corrected for this loss by dividing by .95.

Whereas in non-pregnant patients the estimated plasma volume could be determined from the surface area by reference to the tables of Gibson and Evans (2), in pregnant patients this could not be done. Hence, in the pregnant patients, it was necessary to measure the plasma volume accurately, using the dye T-1824 or Evans blue. This determination was done immediately following completion of the bromsulfalein part of the experiment according to the technique of Price and Longmire (3) with the following modification. Approximately 5 ml. of Evans blue were loaded in a 5-ml. syringe equipped with a guard to keep the plunger steady and weighed together with the needle. The dye was injected into an infusion tubing just above the hub of the infusion's needle and extreme precautions taken to lose none of the dye by using the smallest possible hypodermic needle (gauge No. 26) for injection with a clamp just behind the site of injection to prevent diffusion of the dye backwards into the infusion tubing. Immediately following injection, the infusion

was allowed to run very rapidly for 3 minutes to insure complete flushing of the dye from the infusion tubing. The needle and syringe together with the residual dye on the walls of the syringe were then weighed again and the difference from the original weight represented the amount of T–1824 injected. Samples were taken according to the method of Gibson and Evelyn (4) at 15-minute intervals and the concentration of the dye in the plasma determined in the Coleman spectrophotometer. A constant (K) for the lot of dye used and for the machine was determined from a calibration curve.

RESULTS

A series of non-pregnant women was first examined in order to provide a standard for comparison. This series is presented in Table I; the *EHBF* varied from 1,177 to 1,900 ml. per minute per 1.73 M². of body surface with an average of 1,548 ml. These results are practically the same as those of Bradley and coworkers. It will be noticed that the average ΔP in this series is high. We found it difficult to maintain a constant blood level of the bromsulfalein. Therefore, all our results were dependent on the added determination of plasma volume, and these values were obtained from the tables of Gibson and Evans. The last column in these tables represents the *EHBF* percentage of total blood volume. In these non-pregnant women, it averaged 35 per cent with a range of from 28.2 per cent to 42.8 per cent.

Fifteen normal pregnancies of various periods of gestation were studied and the equivalent hepatic blood flows determined. These data are presented in Table II. The range of *EHBF* was from 1,075 to 2,465 ml. per minute per 1.73 M². of body surface area with an average of 1,554 ml.

TABLE II

The average EHBF in normal pregnancy

Subject	Age in years	Diagnosis	Surface area	Average serum concentration BSP: P	Average serum concentration BSP: H	Average extraction percentage $\frac{P-H}{P}$	Total removal rate BSP	Hepatic blood flow	Hepatic blood flow	Average ΔP	Plasma volume	Hematocrit	Blood volume	*EHBF* per cent of total blood volume
			M.²	*mgm. per cent*		*per cent*	*mgm. per min.*	*ml. per min.*	*ml. per min. per 1.73 M.²*					
M. W.	23	Para-4 Gravida-5 35 wk. gestation	1.83	2.28	1.63	29.4	5.07	1137	1075	.0006340	4480	31.60	6630	17.1
A. K.	27	Para-0 Gravida-1 40 wk. gestation	1.85	1.50	.70	52.6	6.87	1454	1360	.0000466	3533	37.30*	5760	25.2
R. D.	31	Para-3 Gravida-4 8 wk. gestation	1.45	2.59	2.10	18.1	5.63	2065	2465	.0001360	2850	40.20	4670	44.2
L. W.	34	Para-3 Gravida-4 Thr. abortion 12 wk. gestation	1.75	1.61	.89	50.2	7.10	1287	1273	.0001320	3110	36.40	5200	24.7
D. B.	26	5 mo. gestation	1.52	2.09	1.37	34.1	6.42	1319	1500	.0001030	2900	31.40	4270	30.9
A. W.	18	Para-0 Gravida-1 38 wk. gestation	1.65	2.34	1.48	36.1	6.75	1188	1245	.0001160	3080	32.30	4730	25.1
H. S.	32	Hyperemesis gravidarum 3 mo. gest.	1.58	3.02	2.61	14.1	5.67	1646	1803	.0003070	4700	22.40	6300	26.1
K. D.	23	Para-0 Gravida-1 37 wk. gestation	1.80	1.94	1.29	32.6	7.19	1520	1460	.0000205	4500	34.00	7100	21.4
E. K.	25	Para-3 Gravida-4 37 wk. gestation	1.58	3.01	2.15	28.5	6.90	1092	1196	.0002820	3680	31.10	5540	19.7
M. D.	25	Para-4 Gravida-5 Term gestation	1.73	2.12	1.56	26.1	6.49	1854	1854	.0001690	3950	34.80*	6000	30.9
M. M.	38	Thr. abortion 10 wk. gestation	1.57	3.45	2.65	23.0	6.47	1517	1672	.0002140	2350	43.80*	4200	36.1
M. S.	22	6 mo. gestation Para-0 Gravida-1	1.73	1.58	.73	52.4	7.69	1423	1423	.0000930	3380	30.10	4830	29.4
E. S.	21	Para-0 Gravida-1 7 mo. gestation	1.50	2.57	1.71	33.9	7.16	1200	1385	.0002590	3360	32.13	4950	24.2
A. F.	23	Para-1 Gravida-2 7 mo. gestation	1.70	2.61	1.86	28.1	7.86	1697	1728	.0002720	3240	33.80*	4890	34.7
A. P.	24	Bartholin abscess 6 mo. gestation	1.59	1.79	1.30	30.3	6.49	1726	1878	.0001480	3120	31.10	4520	38.1
Average									1554					28.5

* Indicates peripheral vein hematocrit. All others from hepatic vein. (See page 952.)

TABLE III

*The average EHBF in pregnant women with toxemia**

Subject	Age in years	Diagnosis	Surface area	Average serum concentration BSP P	Average serum concentration BSP H	Average extraction percentage $\frac{P-H}{P}$	Total removal rate BSP	Hepatic blood flow	Hepatic blood flow	Average ΔP	Plasma volume	Hematocrit	Blood volume	*EHBF* per cent of total blood volume
			$M.^2$	*mgm. per cent*		*per cent*	*mgm. per min.*	*ml. per min.*	*ml. per min. per 1.73 M.²*					
L. P.	28	Mild pre-eclampsia	1.6	1.85	0.60	62.9	6.72	1007	1090	.0001010	2788	40.6	4640	21.7
E. Z.	26	Essential hypertension	1.68	1.97	1.00	46.8	6.65	1285	1323	.0000520	3060	34.2	4490	28.6
J. M.	20	Severe pre-eclampsia pre-Caesarean	1.6	2.77	2.27	18.3	6.32	1969	2130	.0001399	2263	34.6**	3460	56.9
		10 days post-Caesarean	1.52	2.44	1.84	24.7	5.75	1736	1973	.0002575	2536	27.9	3520	49.3
A. B.	27	Hypertension with superimposed pre-eclampsia	1.68	2.60	2.28	12.4	5.06	2374	2445	.0004154	3413	34.1	5180	45.8
A. G.	28	Nephritis 3 months pregnant	1.76	1.15	0.48	58.3	6.76	1595	1568	.0000761	2286	34.3**	3480	45.8
M. F.	25	Early pregnancy (3rd) with history of eclampsia and severe pre-eclampsia with previous pregnancies	1.82	2.29	1.88	16.2	7.65	4070	3860	.0001327	2625	39.9	4360	88.0

* Table III shows a few toxemic pregnant patients examined with interesting but inconclusive results since there are so few cases.

Patient L. P. with very mild pre-eclampsia had a normal *EHBF*. Patient E. Z. had essential hypertension and a normal *EHBF*. Patient A. G., examined when 3 months pregnant, had chronic nephritis but went on to term successfully; her *EHBF* was normal.

J. M. had severe pre-eclampsia for which Caesarean section was performed; her *EHBF* was somewhat elevated both before delivery and to a less extent post-operatively. It is interesting also to note the high *EHBF* percentage of total blood volume in her case. The same elevations of *EHBF* and *EHBF* per cent of blood volume were found in patient A. B. who had hypertension with superimposed pre-eclampsia, developing premature separation of the placenta and delivering a 6 lb. 11 oz. stillbirth.

Patient M. F. had a very high *EHBF* per minute, so high, in fact, it represented 88 per cent of the total blood volume. Two previous pregnancies had been complicated by eclampsia and pre-eclampsia. She was examined at 10 weeks in this, the third, pregnancy at which time her temperature, pulse, blood pressure and urine were normal. The remainder of her pregnancy was perfectly uneventful, with normal labor occurring at term.

While most inconclusive because of the paucity of examined cases, these results suggest some abnormality of liver blood flow in the severe toxemias of pregnancy. Further study in this direction is expected, and certainly necessary before any definite conclusions can be made as to the effect of toxemias on liver blood flow.

** Indicates peripheral vein hematocrit. All others from hepatic vein. (See page 952.)

which is practically identical with the non-pregnant average *EHBF*. Subject R. D. in this series was examined when she was pregnant 4 months. She had had 2 previous normal pregnancies. Although her *EHBF* was high, 2,465, she went on to term with this pregnancy without developing any signs of toxemia.

As would be expected from the fact that the equivalent hepatic blood flow does not change in normal pregnancy, the *EHBF* percentage of total blood volume was somewhat decreased, particularly late in pregnancy when the blood volume is increased. The *EHBF* percentage of total blood volume in this series averaged 28.5 per cent with a range of 17.1 to 44.2 per cent. A physiological increase in blood volume is a known fact in pregnancy. Liver blood flow remains unchanged, however, presumably because of shunting of the excess blood volume through the placenta.

CONCLUSION

Liver blood flow in normal pregnancy does not differ from liver blood flow in non-pregnant patients with normal livers. The average *EHBF* per minute per 1.73 M.² was 1,554 ml. in normal pregnancy and 1,548 ml. in non-pregnant women. The decreased *EHBF* per cent of total blood volume in pregnancy is an obvious corollary to the fact that liver blood flow remains unchanged in the presence of an increased blood volume of pregnancy.

The authors wish to thank Dr. Stanley E. Bradley for his assistance in establishing this study. We are also indebted to Anna Rosenthal, Edith Oblatt, Marion Heaton, and Margaret Hood for technical assistance.

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BIBLIOGRAPHY

1. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J.J., The estimation of hepatic blood flow in man. J. of Clin. Invest., 1945, 24, 890.
2. Gibson, J. G., and Evans, W. A., Jr., Clinical st of blood volume. II. The relation of plasma total blood volume to venous pressure, blood locity rate, physical measurements, age and sex 90 normal humans. J. Clin. Invest., 1937, 16, 3
3. Price, P. B., and Longmire, W. P., The use of T-1 in plasma volume determinations. Bull. Johns Hop kins Hosp., 1942, 71, 51.
4. Gibson, J. G., Jr., and Evelyn, K. A., Clinical studies of the blood volume. IV. Adaptation of the method to the photoelectric microcolorimeter. J. Clin. Invest., 1938, 17, 153.

EXPERIMENTAL TRANSMISSION OF MINOR RESPIRATORY ILLNESS TO HUMAN VOLUNTEERS BY FILTER-PASSING AGENTS. I. DEMONSTRATION OF TWO TYPES OF ILLNESS CHARACTERIZED BY LONG AND SHORT INCUBATION PERIODS AND DIFFERENT CLINICAL FEATURES[1]

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INTRODUCTION

Investigations of respiratory disease in military populations, and to a less extent in civilian life, have consistently demonstrated that a large majority of the respiratory illnesses are of unknown etiology (1 to 10). Clinical and epidemiological studies have indicated points of similarity and of difference in selected groups of such cases (1, 6, 11, 12), but in the absence of laboratory confirmation, it has been almost impossible to segregate distinct entities. In the Army, for example, studies in the field, in dispensaries, and in the hospital (13, 14) have shown variations in the clinical picture from mild, "coryzal" types of infection, resembling the "common cold," to severe, "grippe-like" illnesses, either with or without pulmonary infiltration. Similarly, epidemiological observations have revealed waves of mild respiratory infections, chiefly resembling the "common cold," in the summer and fall (13) and epidemics of more severe disease, apparently associated with an increased incidence of primary atypical pneumonia, in the winter and spring (2, 13, 15). Laboratory studies have failed to reveal causative agents in these cases, although human volunteer experiments have suggested a relationship between minor respiratory illness and primary atypical pneumonia (16 to 18).

A more direct attempt (*a*) to segregate "entities" from this group of unclassified respiratory illnesses and (*b*) to clarify the relation between minor respiratory illnesses and primary atypical pneumonia has been made by a series of experiments in human volunteers. Four donor subjects were selected whose illnesses appeared clinically to represent different types of respiratory disease:

(*1*) A relatively severe febrile illness, with sore throat, hoarseness and cough, in a recruit; referred to here as "acute respiratory disease" (ARD),

(*2*) A coryzal illness, with fever and constitutional symptoms, in a laboratory officer, referred to here as "severe common cold" (S-CC),

(*3*) A mild coryzal illness, without fever, in a medical officer, referred to here as "common cold" (CC), and

(*4*) A severe febrile illness, with marked constitutional symptoms and rales in the lungs, in a recruit, referred to here as "bronchitis resembling atypical pneumonia" (Br-AP).

Filtered washings from the respiratory tracts of these 4 donors, inoculated into 4 groups of volunteers, induced 2 types of minor respiratory ill-

[1] This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation, and the International Health Division of the Rockefeller Foundation, to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases, for the Commission on Acute Respiratory Diseases.

[2] Members and professional associates of the Commission on Acute Respiratory Diseases were: John H. Dingle, Lt. Col., M.C., A.U.S., Director; Theodore J. Abernethy, Lt. Col., M.C., A.U.S.; George F. Badger, Major, M.C., A.U.S.; Joseph W. Beard, M.D.; Norman L. Cressy, Major, M.C., A.U.S.; A. E. Feller, M. D.; Irving Gordon, M.D.; Alexander D. Langmuir, Major, M.C., A.U.S.; Charles H. Rammelkamp, Jr., M.D.; Elias Strauss, Major, M.C., A.U.S.

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ness as indicated by clinical characteristics and incubation periods. The results are reported in the present paper.

Further differentiation of these 2 types of minor respiratory illness was achieved on the basis of active immunity by reinoculation of the volunteers with homologous and heterologous washings from the donors. Finally, it was determined that primary atypical pneumonia could still be induced in individuals convalescent from both types of disease. These findings are presented in the second paper.

Terminology

Inadequate knowledge of causation of the respiratory illnesses dealt with in this paper results unavoidably in cumbersome nomenclature. The terms and abbreviations employed here are used in an attempt to name the individual illnesses which were selected for study because they might represent entities and to relate those illnesses, insofar as possible, to cases observed in previous investigations.

Undifferentiated acute respiratory disease (ARD). This term is applied to an acute febrile respiratory disease of short duration characterized by constitutional reactions or local respiratory symptoms, or both, of sufficient severity to require hospitalization,[4] and by epidemic occurrence among recruits in the winter months (2, 14, 15). The etiology is unknown. The diagnosis is made by exclusion, based on: (*1*) the absence of clinical or serological evidence of streptococcal infection or specific contagious disease; (*2*) absence of pulmonary consolidation, as determined by roentgenogram; and (*3*) absence of serological evidence of influenza A or B.

Severe common cold (S–CC). This name indicates a respiratory illness characterized by coryza, constitutional symptoms and fever. With respect to the particular donor used in the present experiments, the term was applied retrospectively at the end of the study because of the difficulty in classifying his illness clinically as an instance of "acute respiratory disease" or of "common cold."

Common cold (CC). This term refers to a respiratory infection characterized principally by coryza, minimal constitutional symptoms and little or no fever.

Bronchitis resembling atypical pneumonia (Br–AP). This term is used to describe a febrile respiratory disease in which the character of onset, physical findings, including rales in the lungs, and clinical course of illness are similar to those observed in primary atypical pneumonia; roentgenographic evidence of pulmonary consolidation, however, is lacking (1).

Minor respiratory illness (MRI). This phrase is employed as a general term to indicate those respiratory illnesses, occurring among the recipients in these and previous experiments (16 to 18), which were characterized by variable clinical patterns and absence of roentgenographic evidence of pulmonary consolidation. The term has also been used in previous publications (13, 15) to designate a mild, usually afebrile respiratory infection which was observed among recruits on duty and in dispensaries, but which did not require hospitalization.

METHODS AND MATERIALS

The experiments were performed in the same hotel utilized for previous studies. In general, the selection of volunteers, isolation precautions, clinical and laboratory examinations and inoculation procedures were the same as those employed the year before in the third experiment of the series (18). Each volunteer was isolated in an individual room during the entire experimental period in order to prevent chance exposure to respiratory diseases through outside contact. A control period of isolation of not less than 2 weeks preceded the inoculation of each individual. The techniques of a contagious hospital were instituted; gowns and face masks were worn at all times by the attending staff.

Subjects. Thirty-seven volunteers comprised the first study group. These men were observed during a 7-week period from May 17 to July 5, 1945. At the completion of this study 16 of the original group volunteered to remain in isolation for an additional 7 weeks; the remaining 23 men were released from observation and 25 replacements secured. The second half of the study was conducted during the 7-week period from July 11 to August 31, 1945; 41 volunteers comprised the experimental group. None of the volunteers had participated in previous investigations performed in 1943 or 1944.

Laboratory Studies. Complete blood counts using capillary blood were done at the beginning of the isolation period. Total and differential leukocyte counts were obtained subsequently at 3- or 4-day intervals. The erythrocyte sedimentation rate was determined weekly by the Rourke-Ernstene method (19).

Venous blood was taken at weekly intervals for the following tests: cold hemagglutination (20), antistrep-

[4] In the Army the usual criterion for hospitalization was a temperature of 100° F. or higher.

tolysin (21), heterophile antibody (22) and agglutinin-inhibition tests for influenza viruses A and B (23). From 4 to 6 specimens were obtained from each individual during any given study period. Specimens from each subject were always tested at the same time at the conclusion of the experiment.

Bacteriological study of the throat flora was made on each volunteer and on each member of the attending staff every second day. Swab cultures of the pharynx and tonsils or tonsillar fossae were taken before breakfast, placed immediately in 5 ml. of sterile broth and subsequently plated on blood agar according to a standard technique (24). Every 4th day an additional throat swab was obtained and placed in media selective for the cultivation of *Beta*-hemolytic streptococci (25). The presence or absence and relative frequency of the following organisms were recorded: *Beta*-hemolytic streptococcus, *Staphylococcus aureus*, pneumococcus, *Hemophilus influenzae*, *H. hemolyticus*, Friedländer's bacillus, and gram-negative cocci. The usual mouse technique was employed for the detection of pneumococci. Anaerobic cultures were not performed.

Inocula. The inocula consisted of nasal and pharyngeal washings obtained from each of the 4 donor subjects during the acute stage of illness and from each of nine well persons in a control group. The inocula were prepared in the following manner: Several specimens of nasal and pharyngeal washings in sterile broth were collected from each donor, frozen immediately and stored at $-70°$ C. A few days prior to inoculation, the individual specimens from each donor were thawed rapidly in a 37° C. waterbath and pooled. After manual shaking or homogenization in a Waring blendor, the material was centrifugalized at 4,500 r.p.m. for 10 minutes in an angle centrifuge, and the sediment discarded. Approximately half of the supernate was filtered through Corning "UF" fritted glass filters and half through single-pad Seitz filters. All procedures were carried out in the cold. Specimens from each filter were placed in separate Pyrex Erlenmyer flasks, a portion was reserved for bacteriological study and the balance of the filtrate refrozen and stored at $-70°$ C. until ready for use. Aerobic and anaerobic cultures of the several specimens of filtrate were made on blood-agar plates, and in blood broth and thioglycolate medium. All filtrates proved to be sterile, so that none had to be discarded. On the day of inoculation the specimens were thawed rapidly, kept iced and used in proportion of approximately 1 part Corning filtrate to 1 part Seitz filtrate.

Inoculation procedures. Five groups of men, A, B, C, D and E, were given inoculations of various types of filtrate on different days (Table I). The inoculations were given out-of-doors and sterile precautions were used throughout.

Each volunteer received a total inoculum of 10 ml. administered in 3 equal doses during the morning and afternoon of a single day. Approximately 2.5 ml. was given by nebulizer and 7.5 ml. by atomizer. The material was sprayed into the nose and throat in synchronization with deep inspiration; a tank of nitrogen served as a source of pressure. Comparable techniques were maintained, not only in the separate inoculations of a given day, but also in those given on different days.

Treatment. The treatment of patients was symptomatic. Antipyretic or antibacterial drugs were not used. Nasal instillation of sympathomimetic drugs was avoided. Codeine sulphate and phenobarbital were administered to control headache, cough or restlessness. Barbiturates were used freely as sedatives.

RESULTS

Inoculation of autogenous filtrates (control)

Separate autogenous filtrates were prepared, using pharyngeal and nasal washings collected from each of 9 individuals after 9 and 11 days in isolation, respectively. Such inocula were employed in order (*1*) to assess the mechanical effects of the spraying procedures, (*2*) to demonstrate the possible existence of a latent agent, and (*3*) to avoid the selection, as "control" donors, of apparently normal individuals who might be actually harboring an agent. After a total period of 14 days of isolation, each volunteer was inoculated with his own filtrate (Group A, Table II).

TABLE I

Types of illness in donors of the inocula administered to the volunteers in the various groups

Group	Number of men	Date of inoculation	Types of illness in donors*	Code designation
A	9	5/31/45	None	Control
B	14	6/8/45	"Acute respiratory disease"	ARD
C	14	6/11/45	"Severe common cold"	S-CC
D	10	7/27/45	"Common cold"	CC
E	10	7/26/45	"Bronchitis resembling atypical pneumonia"	Br-AP

* See text for definitions of the illnesses.

TABLE II

Summary of results in 48 volunteers inoculated with filtrates from cases of respiratory disease and in 9 controls inoculated with autogenous filtrates

Group	Type of inoculum*	Days of isolation prior to inoculation	Number of men	Results: MRI	Results: No illness
A	Control	14	9	0	9
B	ARD	22	14	12	2
C	S-CC	25	14	9	5
D	CC	17	10	6	4
E	Br-AP	16	10	4	6

MRI = Minor respiratory illness.
* See Table I and text for explanation of code designations.

Of the 9 volunteers given autogenous filtrates all remained well for a period of 30 days following inoculation (Table II). In no instance were symptoms or physical signs indicative of respiratory disease observed.

Inoculation of ARD filtrate

On June 8, after a control period of 22 days, 14 volunteers received ARD filtrate (Group B, Table II). The donor of this filtrate had suffered a moderately severe respiratory illness comparable to the "undifferentiated acute respiratory disease" of recruits (2, 14, 15). Pertinent data regarding the illness are given below and shown graphically in Figure 1.

The patient (NE) was an 18-year-old soldier with 5 weeks of Army service. He was well until the evening of May 7, at which time he became feverish, drowsy and developed a sore throat. The following morning these symptoms were still present and he was admitted to the hospital. The temperature on admission was 102.8° F., the pulse rate, 124, and the respiratory rate, 24. He appeared moderately ill. Physical examination revealed palpable non-tender cervical lymph nodes, slight nasal discharge (which was no more than normal for the patient), and mild injection of the soft palate, pharynx and tonsils. The tonsils were not conspicuously enlarged; exudate was not present. The lungs were clear. Roentgenograms of the chest taken in posterior-anterior and oblique positions showed no consolidation. Sinus films showed polypoid thickening of both antra, but no evidence of acute infection.

The febrile period was of 2 days' duration. Sore throat and later, hoarseness and cough, were the most prominent symptoms noted. By the 6th day all symptoms had disappeared. Convalescence was uneventful.

The total leukocyte count was normal. Bacteriological studies performed during the first 3 days from throat swabs, and from throat and nasal washings showed *H. influenzae* and *H. hemolyticus*. Serological tests were made on 4 samples of serum obtained at weekly intervals from admission; these tests included cold hemagglutination, heterophile agglutination, antistreptolysin "O" titers and antibody determinations for influenza viruses A and B. None of these tests revealed findings of diagnostic significance.

Of the 14 volunteers inoculated with ARD filtrate, 12 developed minor respiratory illness and 2 remained well (Table II). None of the group developed primary atypical pneumonia.

In 9 individuals, symptoms were first observed on the 5th or 6th day after inoculation. In the remaining 3 cases, onset of illness occurred on the 3rd, 8th and 9th days, respectively.

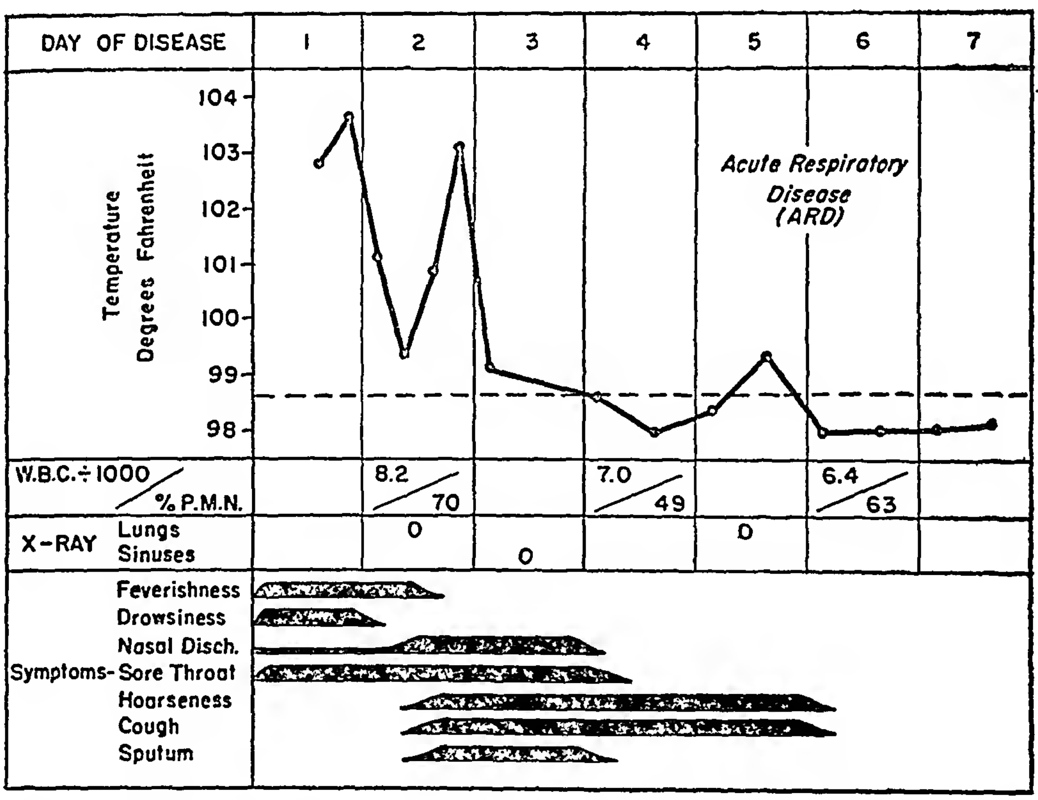

FIG. 1. CLINICAL CHART OF DONOR WHOSE ILLNESS WAS TERMED ACUTE RESPIRATORY DISEASE (ARD)

The clinical pattern observed in these cases was variable. In general the illnesses were mild in nature, with minimal constitutional symptoms, and varying degrees of symptoms and signs referable to upper respiratory tract. In 7 patients the duration of illness was no more than 4 days; in 5 patients it lasted between 6 and 12 days. Fever was not a conspicuous feature in any of the subjects; none had as brisk a febrile response to illness as did the donor.

The onset of illness was usually characterized by the development of dry or sore throat during the course of 24 or 48 hours. Generally, there were accompanying signs of mild inflammation of the soft palate or pharynx (injection or lymphoid hyperplasia). Nasal symptoms (sneezing, obstruction and discharge) were frequent during the first 48 hours from onset, but were of minimal intensity and not associated with obvious signs of nasal obstruction or coryza. Hoarseness and cough were observed at some time by approximately half of the patients, but were not conspicuous features of the early course of illness.

The following case reports illustrate the characteristics of the illnesses following inoculation of ARD material.

Case No. RP3-331. Five days after inoculation this patient first observed sore throat which was unaccompanied by constitutional or other local symptoms. During the next 24 hours there was minimal nasal obstruction and discharge as well as soreness of the throat. Physical examination revealed only prominent pharyngeal lymphoid follicles. On the 3rd day of illness he complained of headache; there was improvement in nasal symptoms, but no lessening of throat symptoms. The cervical lymph nodes were tender, but not conspicuously enlarged. Nasal obstruction was of moderate degree. There was gradual improvement in symptoms and signs, and all evidences of illness disappeared by the 7th day. The maximum temperature was 99.8° F. on the day after onset of symptoms, or 6 days after inoculation.

The total and differential leukocyte counts showed no alteration following inoculation. There was no elevation of the erythrocyte sedimentation rate at any time. Serological procedures showed no diagnostic increase in antibodies to influenza viruses A or B, heterophile agglutinins, or cold hemagglutinins. *Beta*-hemolytic streptococcus, group C, was isolated from throat cultures before and after inoculation, but no diagnostic rise of antistreptolysins was demonstrated in the patient's serum during convalescence.

Case No. RP3-321. This 26-year-old subject gave a history of infrequent mild respiratory infections of short duration occurring once a year, and consisting of sore throat, nasal congestion and coryza. He had had no respiratory illness in the previous 8 months. Beginning 6 days after inoculation he observed gradual onset of dryness of the throat, which was later followed by soreness of the throat, dry cough and malaise. On the 2nd day of illness he complained of feverishness, loss of appetite, aching of the eye-balls, sneezing and "stuffiness" of the nose. Physical examination revealed tender cervical lymph nodes and minimal injection of the nasal and pharyngeal mucosa. On the 3rd day there was improvement in constitutional symptoms but little change in throat or nasal symptoms; in addition the cough became productive of mucoid sputum. From the 4th to 8th day of illness he noted substernal discomfort during coughing. The nasal symptoms persisted until the 10th day. Cough decreased in amount and intensity, finally disappearing on the 12th day, 18 days after inoculation. Pulmonary infiltration was not demonstrated by auscultation or by roentgenography. Roentgenograms of the paranasal sinuses showed no evidence of acute or chronic infection. Recovery was uneventful. Bacteriological examinations showed no change in flora of the throat; serological examinations were non-contributory.

Inoculation of S–CC filtrate

Fourteen volunteers were inoculated with S–CC filtrate after a control isolation period of 25 days (Group C, Table II). The donor of this inoculum had experienced a moderately severe febrile respiratory illness in which nasal symptoms, coryza and cough were associated with constitutional symptoms. The clinical course of illness in this donor is given below and in Figure 2.

OL, a 35-year-old laboratory officer, had been well for at least a month when on the morning of April 23 he observed coryza, cough and substernal soreness. He reported for duty but felt unwell because of malaise, fever, coryza and continued cough which was productive intermittently of greenish-yellow sputum. In the afternoon, the malaise became worse. By evening he felt "grippy" and observed a dull frontal headache. The oral temperature was 100.2° F. The following morning there were dryness of eyes, mouth, throat and lips and diminution of nasal symptoms. Physical examination revealed a temperature of 99.4° F., mild hypertrophy of the pharyngeal lymph follicles, edema and injection of the nasal mucosa without discharge. The lungs were clear. Roentgenograms of the lungs and paranasal sinuses showed no acute or chronic pathological changes. Trans-illumination of the sinuses was normal; a consultant found no evidence of allergic rhinitis. The temperature rose to 101.2° F. on the evening of April 24, the 2nd day.

On the morning of the 3rd day the most prominent symptoms were malaise, feverishness, headache, anorexia, obstruction of the nares and cough. Physical examination revealed profuse serous nasal discharge. By the 4th day he was considerably improved; coughing was less fre-

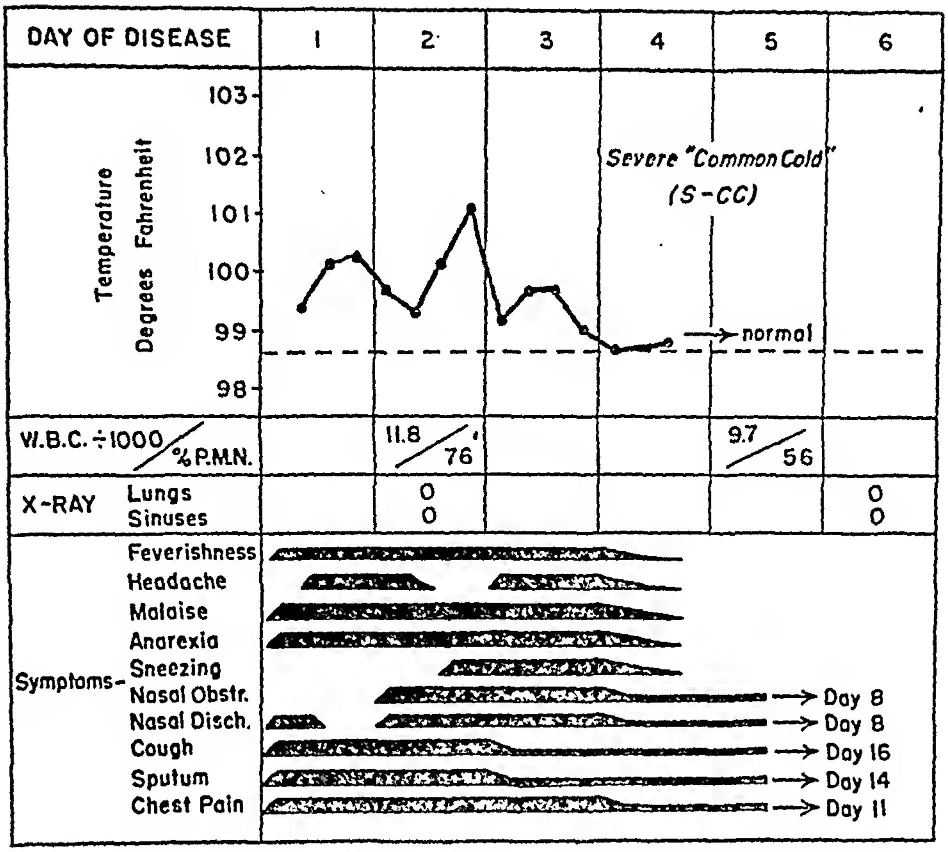

Fig. 2. Clinical Chart of Donor Whose Illness was Termed Severe "Common Cold" (S–CC)

quent and there was gradual decrease in nasal symptoms and signs. Roentgenograms of the chest and sinuses on the 6th day showed no pathological changes. Convalescence was prolonged.

The total leukocyte count on the 2nd day was 11,800; on the 5th day the count had decreased to 9,700. Polymorphonuclear leucocytes constituted 76 and 56 per cent, respectively, of the differential formulae. Cultures of the throat and sputum revealed pneumococcus, type 11. *H. hemolyticus* was isolated from throat washings. Samples of acute and convalescent sera showed no increase in antibody titer to sheep cells, streptolysin "O," group "O" human erythrocytes, or influenza viruses A and B.

Of the 14 subjects given S–CC inoculum, nine became ill with mild to moderately severe respiratory infection (Table II). None of this group developed roentgenographic evidence of primary atypical pneumonia.

In contrast with the finding in the ARD group, all patients developed illness within 24 to 48 hours after inoculation of S–CC filtrate. The clinical pattern was featured by sneezing, nasal obstruction and objective evidence of coryza. Because of the prominence of nasal symptoms and signs there was usually little difficulty encountered at the bedside in distinguishing this type of illness from that which followed the ARD inoculum. The duration of illness, furthermore, was more prolonged. Symptoms were present for from 4 to 7 days in 4 patients, from 9 to 13 days in 5. The type and course of illness resulting from S–CC inoculum resembled that of naturally acquired cases of the "common cold." An example is presented in the following case report:

Case No. RP3–236. This 22-year- old volunteer had experienced rare head colds of 2 weeks' duration during the past few years. He suffered occasionally from frontal headaches and, during most of the preceding year, from slight nasal congestion and post-nasal discharge. His last previous respiratory infection occurred during the spring of 1945 with symptoms lasting for only 1 day. During the 25-day control period of isolation he was free of respiratory symptoms.

Inoculated on June 11, he first noted an abrupt onset of illness at 6 p.m. on June 12. The incubation period was calculated to be not less than 27 hours nor more than 33 hours. The initial symptoms were excessive sneezing, nasal discharge and increased post-nasal drip. Later that evening he noted generalized weakness, dull cranial discomfort and mild sore throat. The following day cough and feverishness were present, and physical examination disclosed only slight injection of the pharynx. The temperature rose to 100.2° F. on the afternoon of the 2nd day

of illness. On the 3rd day (June 14) the temperature returned to normal, headache persisted, but other constitutional symptoms were absent. Local symptoms, however, were prominent and consisted of sneezing, nasal obstruction and discharge, dry and sore throat, hoarseness and dry cough. Physical examination at this time revealed nasal obstruction, thin serous discharge from the nares and slight injection of the pharynx. The lungs were clear. The subsequent course of illness was characterized by improvement in nasal symptoms and signs and gradual diminution of cough which was no longer present after the 13th day. Roentgenograms of the chest showed no pulmonary infiltration at any time. Sinus films were clear before as well as 2 weeks after inoculation.

The leukocyte counts, differential formulae, and erythrocyte sedimentation rates were within normal limits. Serological tests on acute and convalescent sera showed no antibodies which were diagnostic of influenza, infectious mononucleosis, primary atypical pneumonia or streptococcal infection. Cultures of the throat showed *Beta-hemolytic* streptococcus, Group D, and pneumococcus, type 3, both of which were isolated repeatedly before as well as after inoculation.

Inoculation of CC filtrate

After an isolation period of 17 days, 10 volunteers were given CC filtrate obtained from a donor who had a mild afebrile respiratory illness characterized principally by coryza. This illness was considered to resemble the "common cold" (Group D, Table II). During convalescence from his infection the donor developed cold hemagglutinins in the serum in low titer. The course of illness in this patient is shown in the following case report and in Figure 3.

MI, a 29-year-old medical officer, first observed onset of respiratory disease on May 11, 1945. The first symptoms were dry throat and slight burning and irritation of the nose. Later in the day there was watering of the eyes. The following morning sneezing, nasal obstruction and coryza were prominent symptoms. The nasal discharge was copious and serous in character. Mild dysphonia also was present for a short period. Constitutional symptoms, except for fatigability, were absent. He carried on with his normal duties. Physical findings were confined to congestion and edema of the nasal mucosa. Roentgenograms of the chest and sinuses were interpreted as being normal.

The maximum temperature did not exceed 99° F. Nasal obstruction and discharge were the most prominent features of illness. The appearance of the nasal mucosa did not suggest an allergic rhinitis. Cough was present for 2 days but was non-productive. No rales were audible in the lungs at any time.

A single total leukocyte determination showed 10,500 cells with normal differential distribution. Cold hemag-

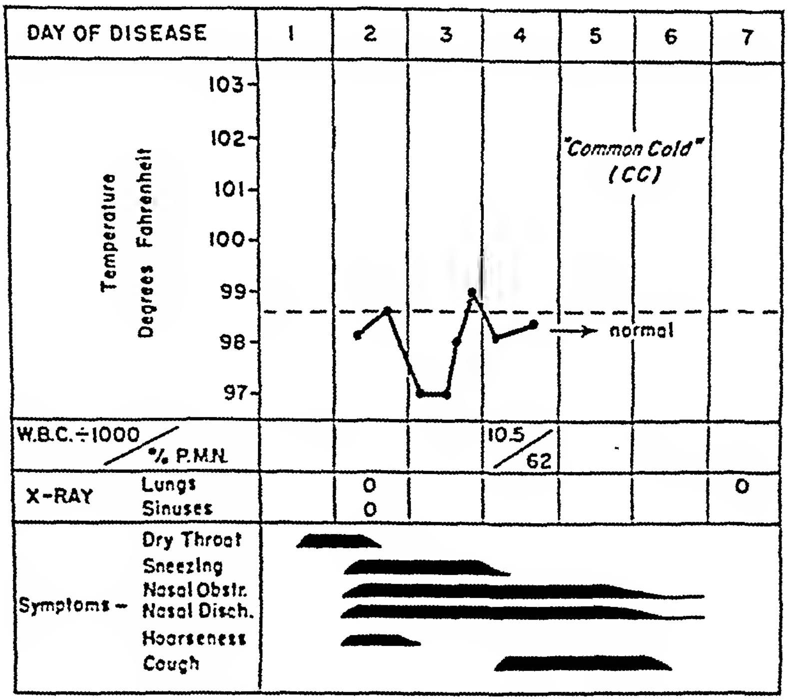

FIG. 3. CLINICAL CHART OF DONOR WHOSE ILLNESS WAS TERMED "COMMON COLD" (CC)

glutinin titers were as follows: 2nd day, < 8; 9th day, 32; 16th day, < 8; 23rd day, 8. Antistreptolysin, heterophile and influenza antibody studies showed no diagnostic increases in titer.

Six of the 10 subjects became ill following the inoculation of CC filtrate (Table II). The illnesses were not associated with fever, were mild in character and were featured by the presence of nasal symptoms and signs resembling those of the "common cold." None of the patients developed pulmonary infiltration or cold hemagglutinins.

Onsets of illness occurred approximately 24 hours after inoculation in 3 cases, on the 2nd day in 2 cases, and the 4th day in 1 case. Illness in these patients usually began abruptly with the development of sneezing, nasal obstruction, and coryza in association with constitutional symptoms of mild degree. In 2 patients, dry or sore throat and cough were also early symptoms. In 2 patients, hoarseness was noted. Constitutional symptoms of chilliness, feverishness, headache, malaise or weakness were observed by 5 of the 6 cases but were of short duration.

Abnormal physical signs in the respiratory tract, except for obvious nasal obstruction and discharge, were minimal. Injection of the soft palate was not encountered and signs of inflammation of the pharynx (injection or lymphoid hyperplasia) were present in 3 cases. Rales were not audible. The illness in one patient, representative of the group, is given in the following case report.

Case No. RP4–321. A 30-year-old male had had "influenza" in January 1944. He usually experienced 2 to 3 respiratory infections each winter lasting about 1 week and manifested by sore throat, nasal congestion and coryza. There had been no illness of this type in the previous 4 months. During the pre-inoculation period of isolation he was well.

On July 28, at 2 p.m., approximately 30 hours after the first inoculation, he noted abrupt onset of excessive sneezing, nasal obstruction and coryza. Later that day he observed myalgia and postorbital aching. Dry cough and hoarseness were intermittently present during the first 12 hours. The following day, in addition to the above symptoms, he observed headache, a dry, sore throat and substernal aching. Physical examination revealed moderate nasal obstruction and clear serous discharge.

On the 3rd day (June 30) the constitutional symptoms were no longer present, the nasal symptoms were unchanged, post-nasal "drip" was noted and the cough was productive of a slight amount of mucoid sputum. Prominent lymphoid follicles on the pharynx were observed for the first time on this date. The subsequent course was characterized by rapid subsidence of all symptoms and signs. The clinical course of illness lasted 5 days. There were no signs of pulmonary infiltration. Recovery was uneventful.

The total and differential leukocyte counts and erythrocyte sedimentation rate showed no significant change from normal following inoculation. Cold hemagglutinins were not present in significant titer, all specimens showing titers of < 8. Bacteriological study of the throat flora showed no significant change as a result of inoculation or of illness.

Inoculation of Br-AP filtrate

After a control isolation period of 16 days, a group of 10 volunteers was given filtrate Br-AP (Group E, Table II). The illness in this donor was more severe than that experienced by any of the other donors. The symptoms, physical signs, clinical course and serological findings were consistent with the diagnosis of primary atypical pneumonia. Roentgenographic evidence of pulmonary infiltration, however, was lacking. The course of illness is given in the following case history and shown graphically in Figure 4.

BA, a 35-year-old soldier with 1 month of Army service, was admitted to the hospital on April 30, 1945. On the afternoon of admission there was an abrupt onset of chilliness, feverishness, generalized aching, weakness and dry cough. Later, headache was noted. The temperature was 100.8° F., the pulse, 80, and respirations, 20 per minute. Physical examination the next day revealed a moderately ill patient whose soft palate and pharynx were injected. The lungs at this time were clear to percussion and auscultation and roentgenograms of the chest and sinuses were normal. During the 2nd day of disease the temperature rose to a maximum of 103.5° F., the constitutional symptoms continued, cough persisted and was productive of a small amount of thick yellow sputum. On the 3rd day, the patient was still moderately ill, the temperature reaching a maximum of 104° F. Fine rales were audible for the first time at the base of the left lung. A roentgenogram of the chest on the 4th day was clear. Rales continued to be heard at the left base, and subsequently, at the right base, until the 6th day. The temperature fell by lysis, reaching normal on the 6th day. Cough and sputum continued until the 10th day.

The total leukocyte and differential counts were normal. Throat cultures showed *Hemophilus influenzae* and *H. hemolyticus*. From throat washings and sputa, the following organisms were isolated: pneumococcus, types 10, 14 and 29; *H. influenzae* and *H. hemolyticus*. Cold hemagglutinins were present in the convalescent sera during the 3rd week in a titer of 16; blood taken during the acute illness showed a titer of < 8. Heterophile antibody tests, antistreptolysin determinations and agglutinin-inhibition tests for influenza viruses A and B showed no diagnostic increases in titers.

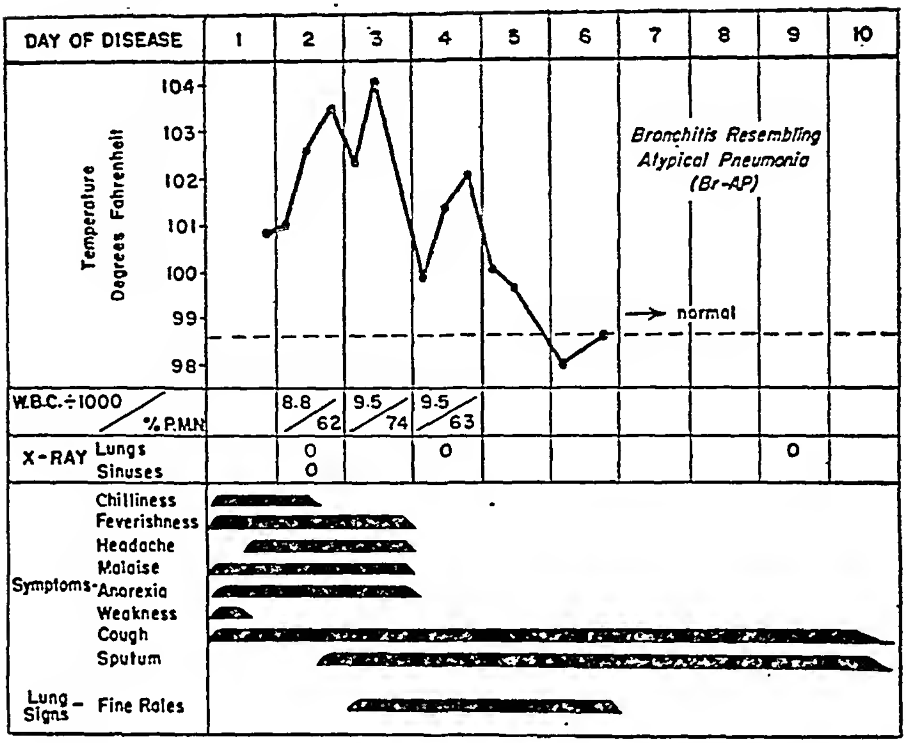

FIG. 4. CLINICAL CHART OF DONOR WHOSE ILLNESS WAS TERMED BRONCHITIS RESEMBLING ATYPICAL PNEUMONIA (Br–AP)

Of the 10 volunteers receiving Br–AP filtrate, 4 developed minor respiratory illnesses (Table II). Onsets of disease in these patients were observed 2, 3, 3 and 7 days, respectively, after inoculation. The illnesses were mild; fever was not observed in any of the patients. Auscultatory or roentgenographic evidence of pulmonary infiltration was not detected in any of those with illness.

Constitutional symptoms of feverishness, headache and malaise were present in only 1 patient. Two patients experienced sore throat as the initial symptom; the remaining 2, nasal symptoms. Hoarseness was noted in 2 cases. Cough was not observed in any of the 4 who became ill.

Physical findings were minimal and consisted of tender cervical lymph nodes (1 case), nasal injection and obstruction (2 cases), pharyngeal injection (1 case) and pharyngeal lymphoid hyperplasia (2 cases).

No characteristic clinical pattern was evident in these cases. None of the illnesses was as severe as that of the donor of the inoculum.

CLINICAL CORRELATIONS

Comparison of the types of illness observed in each of the experimental groups revealed many clinical features which were common to all groups. Nevertheless, certain features such as nasal symptoms and signs, were more prominent in some groups than others. For purposes of increasing the material available for analysis, 6 cases of illness from the second half of the study are included (26). Four of these illnesses were induced by reinoculation of 5 men of the control group with filtrate S–CC, and the other 2 illnesses followed the inoculation of 5 men with filtrate ARD (see Figure 1 in the following paper [26]). Thus, clinical correlations were made from the following material: 14 cases, filtrate ARD; 13 cases, filtrate S–CC; 6 cases, filtrate CC; and 4 cases, filtrate Br–AP.

Incubation period. The sharp difference in incubation periods observed in the groups inoculated with filtrate ARD and S–CC is readily seen in Figure 5. Although the number of cases in the CC

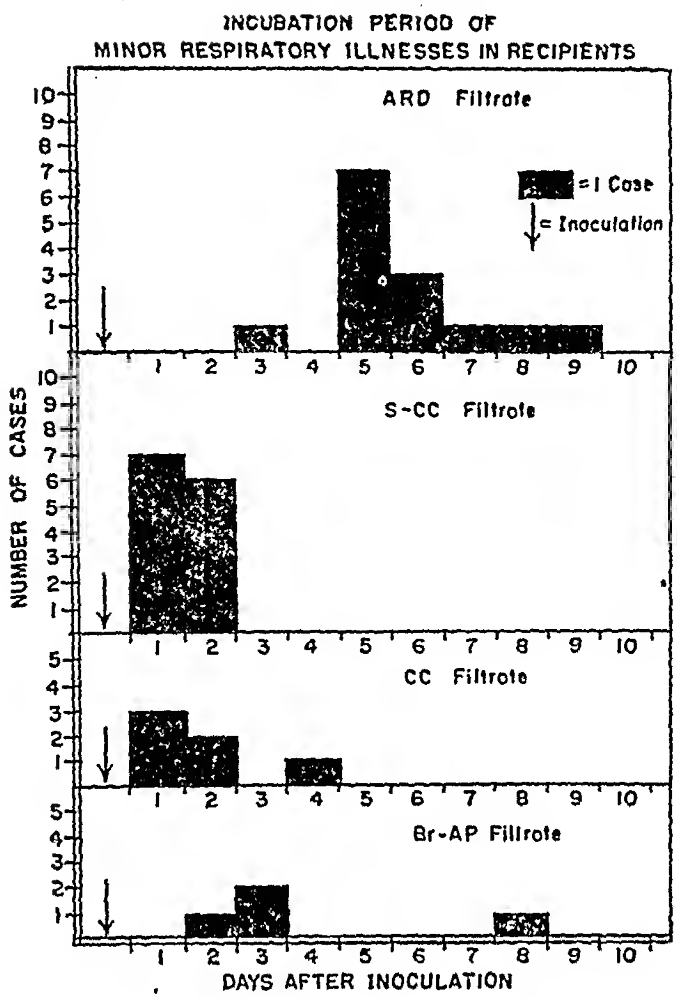

Fig. 5. Incubation Period of Minor Respiratory Illnesses in Recipients of the 4 Inocula

See Table I and text for explanation of code designations ARD, S-CC, CC, and Br-AP.

TABLE III

Total frequency of symptoms in 37 cases of experimentally induced minor respiratory illness

Symptom		Frequency of symptoms in various groups*			
		ARD (14 cases)	S-CC (13 cases)	CC (6 cases)	Br-AP (4 cases)
Constitutional:	Chilliness	0	0	1	0
	Feverishness	1	4	3	1
	Headache	7	8	4	1
	Malaise	4	3	4	1
	Anorexia	3	1	0	0
	Weakness	2	3	1	0
Local:	Sneezing	4	8	5	1
	Nasal obstruction	10	11	6	2
	Nasal discharge	8	12	6	2
	Dry throat	6	8	5	0
	Sore throat	10	7	2	3
	Hoarseness	5	8	2	2
	Cough	6	9	3	0
	Sputum	5	4	1	0
	Chest pain	3	4	2	0

* See Table I and text for explanation of code designations.

group is small, 5 of the 6 cases had onsets on the 1st or 2nd day after inoculation. In this respect and in other features which will be pointed out below, the CC and S-CC groups were similar. The incubation period in the Br-AP group was not clearly defined because of the small number of illnesses.

Symptomatology. The total frequency of symptoms is shown in Table III. Only slight differences are apparent for each symptom among the various diagnostic groups. With respect to daily frequency and time of appearance of nasal and throat symptoms, however, further analysis revealed certain differences between the ARD and S-CC groups (Figure 6). Nasal symptoms (sneezing, obstruction and discharge) tended to develop earlier, that is, within the first 48 hours from onset and were present in greater day-by-day

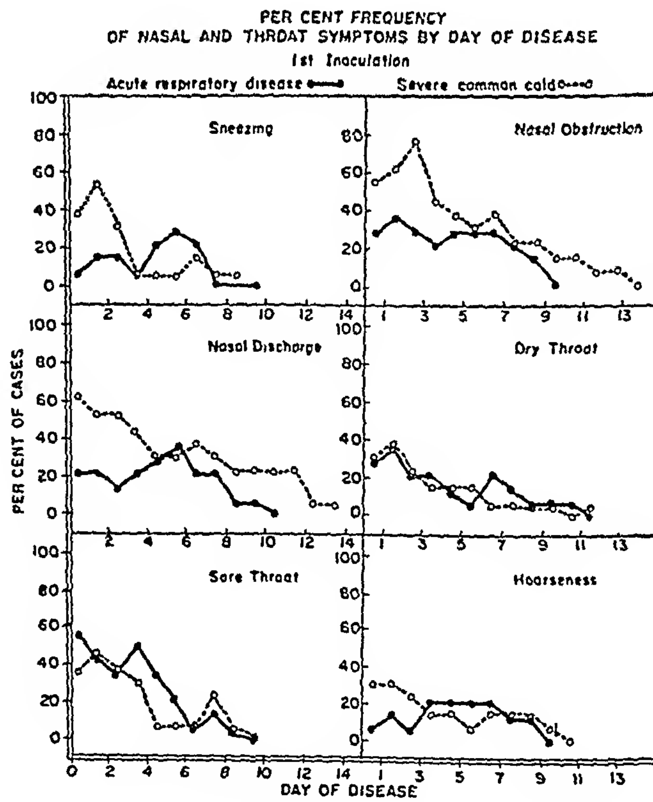

Fig. 6. Daily Frequency and Time of Appearance of Nasal and Throat Symptoms in Minor Respiratory Diseases Experimentally Induced by ARD and S-CC Inocula

frequency, in the S–CC group than in the ARD group. These findings substantiated clinical impressions gained at the time the patients were ill. Throat symptoms (dry and sore throat), on the other hand, appeared in each group at approximately the same time and were present in the same daily frequency during the course of disease. The CC and Br–AP groups are omitted from consideration because of the small number of cases.

The number of nasal symptoms (sneezing, obstruction and discharge) observed during the first 48 hours also showed differences among the groups (Table IV). The presence of 2, or of all 3 nasal symptoms was encountered more often in those men in the S–CC and CC groups. Conversely, multiple nasal symptoms were experienced infrequently by those in the ARD group.

TABLE IV

Total number of nasal symptoms present within 48 hours after onset in various diagnostic groups

Number of nasal symptoms*	Group†		
	ARD	S-CC	CC
None	4	3	0
One	7	3	0
Two	3	1	2
Three	0	6	4
Total cases	14	13	6

* Sneezing, obstruction and discharge.
† See Table I and text for explanation of code designations.

The duration of throat complaints and nasal symptoms was approximately the same in the various groups. Obstruction and discharge tended to persist somewhat longer in the S–CC and CC groups.

The relation of nasal symptoms to nasal signs and throat symptoms to signs during the first 2 days of illness is shown in Table V. In general, better correlation was found between nasal symptoms and signs in groups S–CC and CC, in which coryza was a prominent feature, than occurred in group ARD. Conversely, more correlation was found between throat symptoms and signs in the ARD group, in which sore throat was a characteristic feature, than was shown in the other groups.

Physical signs. The total frequency of physical signs at any time during illness is shown in Table VI. It is apparent that nasal signs were frequent

TABLE V

The relation of nasal symptoms and signs and throat symptoms and signs during first 48 hours of illness in various diagnostic groups

Physical signs	Symptoms*					
	ARD§		S-CC§		CC§	
	Present	Absent	Present	Absent	Present	Absent
Nasal symptoms						
Nasal signs†						
Present	2	2	5	2	4	2
Absent	8	2	5	1	0	0
Throat symptoms						
Throat signs‡						
Present	7	1	1	0	0	0
Absent	4	2	8	4	4	2

* Symptoms: Nasal = Sneezing, obstruction or discharge. Throat = Dryness or soreness.
† Nasal signs = Injection, obstruction or discharge.
‡ Throat signs = Injection of a soft palate, injection of pharynx or lymphoid hyperplasia of pharynx.
§ See Table I and text for explanation of code designations.

and that signs referable to the throat were minimal. The percentage frequency of nasal and throat signs of any degree by day of disease is shown in Figure 7 for groups ARD and S–CC. The other 2 groups are omitted from this analysis because of the small number of cases. Differences between the 2 groups were most marked in the case of nasal obstruction and discharge.

TABLE VI

Total frequency of physical signs in 37 cases of experimentally induced minor respiratory illness

Physical signs	Frequency of physical signs in various groups*			
	ARD (14 cases)	S-CC (13 cases)	CC (6 cases)	Br-AP (4 cases)
Nasal: Injection	8	6	1	2
Obstruction	8	10	5	2
Discharge	10	11	6	1
Soft palate: Injection	4	1	0	0
Pharynx: Injection	5	3	1	1
Lymphoid hyperplasia	5	3	2	2
Exudate	0	0	0	0
Cervical lymph nodes: Enlarged	2	0	0	0
Tender	5	3	1	1

* See Table I and text for explanation of code designations.

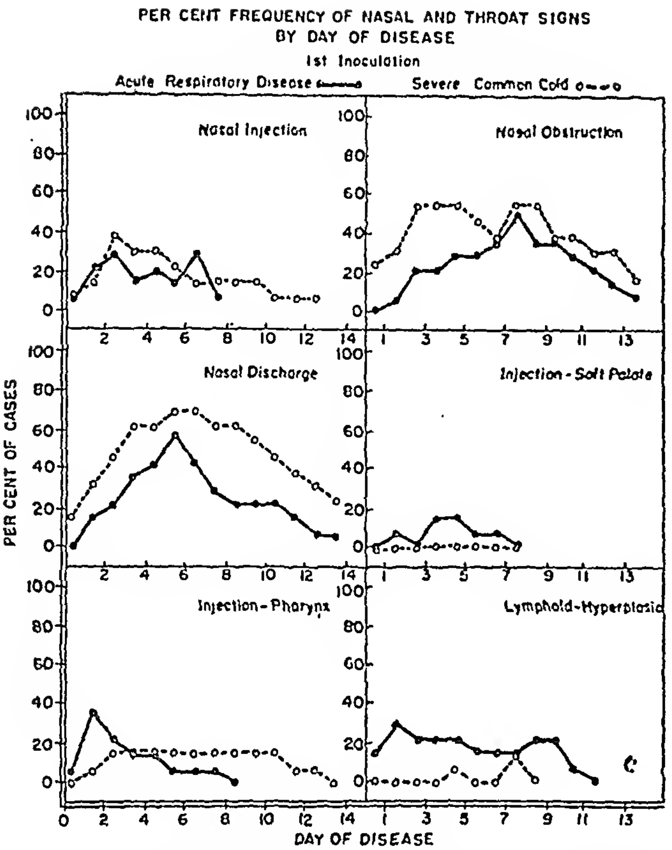

FIG. 7. DAILY FREQUENCY AND TIME OF APPEARANCE OF NASAL AND THROAT SIGNS IN MINOR RESPIRATORY DISEASES EXPERIMENTALLY INDUCED BY ARD AND S-CC INOCULA

Fever. Fever was not a conspicuous clinical feature of illness in the majority of the volunteers (Table VII). Only 2 individuals, both of whom received S-CC inoculum, developed temperatures of 100° F. or more. Maximum temperatures in the other groups did not exceed 99.8° F. The median febrile curves were essentially normal in the ARD and S-CC groups. Slightly higher temperatures, however, were found in the ARD group from the 5th to the 8th days, and in the S-CC group on the 2nd day. These periods corresponded with the time when symptoms were present in the two groups of cases.

TABLE VII

Febrile response in 37 cases of experimentally induced minor respiratory illness

Group	Total cases with illness*	Number of cases with			Maximum fever at any time (degrees F.)
		No fever 99° F.	Fever 99.2–99.8°	Fever 100° F.+	
ARD	14	5	7	0	99.8
S-CC	13	3	7	2	100.2
CC	6	4	1	0	99.4
Br-AP	4	1	3	0	99.8

* 4 cases (2 in the ARD, 1 in the S-CC group and 1 in the CC group), showing intermittent low grade fever (99 to 99.4° F.) before and after inoculation, are omitted from analysis. In each case the temperature curve was not altered as a result of inoculation.

TABLE VIII

Average total leukocyte count in 57 volunteers in relation to inoculation and in 31 patients with illness in relation to onset

Type of inoculum*	Number inoculated	Leukocyte count (thousands)		Number with illness	Leukocyte count (thousands)	
		Before Inoculation	After Inoculation		Before onset	After onset
Autogenous	9	7.3	7.1	0		
ARD	14	7.6	6.7	12	7.6	6.5
S-CC	14	7.7	7.0	9	7.6	7.3
CC	10	7.0	7.5	6	6.9	7.4
Br-AP	10	8.0	7.6	4	8.7	8.0

* See Table I and text for explanation of code designations.

LABORATORY STUDIES

Hematology. Tabulation of the average total leukocyte count was made in each of the experimental groups according to inoculation and in relation to onset. A total of 381 individual leukocyte counts was made during the first study. As shown in Table VIII, the leukocyte counts in 3 of the 4 groups (ARD, S-CC and Br-AP) and in the control group fell to a slight extent; the average count in the remaining group (CC) increased. Similar differences were shown when the analyses were restricted to patients with illness. All of the average counts, however, were normal and within the limits of technical variation. Individual instances of leukocytosis or leukopenia were not encountered.

The relative percentage of polymorphonuclear neutrophils, lymphocytes, eosinophils and monocytes showed no significant change in any group either as a result of inoculation or after the development of illness.

The corrected erythrocyte sedimentation rate was normal (0.2 to 0.4) in every subject both before as well as after inoculation.

Serology. A diagnostic increase in titer (4-fold or greater) of cold hemagglutinins was not encountered following inoculation in any of the subjects. Similarly, no increase in titer of hetero-

phile antibodies, or of antibodies against streptolysin "O" and influenza viruses A and B was shown in representative samples of serum.

Bacteriology. The results of bacteriological study of the throat flora, performed at 2-day intervals, were analyzed for the purpose of detecting any change in flora for any given organism in relation to time of inoculation or resultant illness. The organisms which formed the basis of the analysis were the *Beta*-hemolytic streptococcus, pneumococcus, *H. influenzae* and *H. hemolyticus.*

During the period of May 18 to 31, 37 individuals comprising the control, ARD, and S–CC groups were in quarantine prior to inoculation. After May 31, when the control group received autogenous filtrates the other 2 groups were observed continuously and on June 8 and 11, respectively, they received initial inoculations. Thus, the interval, May 18 to 31, provided a suitable period in which the bacteriological results could be compared within each of the groups. The interval, June 2 to 8, was a period in which the bacteriological findings in the inoculated control group could be contrasted with those in the ARD group; similarly, comparisons were permitted between the control and S–CC groups during the period, June 2 to 10. The results are given in Table IX. Marked fluctuations occurred in the frequency with which each of the 4 organisms was isolated during the period of quarantine, May 18 to 31. No significant change in throat flora was observed in the control group as a result of having received autogenous filtrate.

The effect of inoculation on the pharyngeal flora of the ARD and S–CC groups is compared in Table X. The analysis was restricted to those individuals who experienced illness, and the results are contrasted with those obtained in the control group, the members of which had experienced no illness. It is apparent that before inoculation there was wide fluctuation in the percentage of individuals from whom any one of the organisms was isolated. Similar variations were evident after inoculation. Comparison of the results in the ARD and S–CC groups after inoculation with those of the control group showed slight differences; for example, in the case of the pneumococcus. However, these differences were of no greater order of

TABLE IX

*Bacteriological results in all individuals during period of quarantine and after inoculation of control group**

Date	Per cent of individuals harboring specified organisms											
	B. H. S.†			Pneumococcus			*H. influenzae*			*H. hemolyticus*		
	Control	ARD	S-CC‡	Control	ARD	S-CC	Control	ARD	S-CC	Control	ARD	S-CC
5/18	22	9	8	11	27	25	22	18	0	44	27	42
19	22	9	25	33	8	25	0	27	8	22	18	25
20	0	0	0	33	43	33	0	14	0	0	7	0
21	25	7	25	45	36	25	12	36	58	50	21	25
23	11	21	33	33	36	8	11	38	33	67	0	42
25	11	14	21	33	43	50	22	23	29	56	36	28
27	11	31	7	67	57	43	0	8	29	44	54	50
29	11	23	7	62	57	29	22	38	57	67	43	57
31	11	27	23	56	71	57	11	27	46	78	36	54
Inoculation of control group with autogenous filtrate (5/31)§												
6/2	*25*	17	8	*45*	71	62	*12*	17	33	*75*	41	67
4	*12*	15	15	*33*	50	43	*12*	31	23	*100*	62	62
6	*22*	7	7	*67*	50	43	*0*	29	14	*11*	25	14
8	*22*	21	21	*45*	36	43	*0*	7	21	*44*	7	25
10	*11*		15	*45*		64	*22*		31	*67*		31

* Figures represent specified percentage of individuals in any particular group showing the presence of specified organism on each day. The presence of *Beta*-hemolytic streptococcus, *H. influenzae* and *H. hemolyticus* was identified on blood agar plates. Only the results of isolation of pneumococci by mouse inoculation are recorded.

† B. H. S. = *Beta*-hemolytic streptococcus.

‡ Control group = 9 individuals; ARD group = 14; S-CC group = 14; see Table I and text for explanation of code designations.

§ Figures in italics indicate results in inoculated group.

TABLE X

*Bacteriological results in those individuals developing illnesses, before and after inoculation with the ARD and S-CC filtrates, and in the control group**

Date	Per cent of individuals harboring specified organisms											
	B. H. S.*			Pneumococcus			*H. influenzae*			*H. hemolyticus*		
	Control	ARD	S-CC†	Control	ARD	S-CC	Control	ARD	S-CC	Control	ARD	S-CC
5/18		10	12		30	25		20	0		30	25
19		11	38		10	12		33	12		22	0
20		0	0		42	38		17	0		8	0
21		8	38		42	12		42	50		25	12
23		17	38		33	0		46	37		0	37
25		8	33		42	33		27	33		33	33
27		27	11		50	33		10	33		55	45
29		17	11		50	11		46	56		33	56
31		33	25		58	56		33	62		67	50
6/2		20	12		67	50		20	37		40	62
4		18	22		42	45		36	11		73	56
6		8	11		42	33		33	11		25	11
8	22	17	33	45	33	45	0	8	0	44	8	33
Inoculation of ARD group (6/8)*												
10	11	*0*	33	45	*25*	56	22	*36*	22	67	*18*	33
Inoculation of S-CC group (6/11)												
12	33	*8*	*33*	22	*36*	*67*	11	*17*	*0*	33	*17*	*11*
14	22	*25*	*22*	45	*58*	*67*	22	*33*	*22*	33	*25*	*45*
16	22	*25*	*57*	38	*42*	*45*	11	*17*	*57*	56	*50*	*43*
18	22	*10*	*11*	56	*33*	*45*	11	*40*	*33*	56	*30*	*33*
20	22	*17*	*33*	56	*42*	*56*	22	*33*	*44*	67	*25*	*56*
22	12	*17*	*33*	56	*25*	*56*	0	*58*	*56*	62	*50*	*45*
24	14	*18*	*11*	22	*67*	*56*	14	*36*	*22*	57	*46*	*67*
26	0	*0*	*12*	56	*50*	*78*	17	*50*	*12*	50	*25*	*75*
28	0	*9*	*11*	89	*50*	*67*	11	*18*	*11*	11	*9*	*33*

* For explanation of figures and abbreviations, see Legend, Table IX.
† Control group = 9 individuals; ARD group = 12; S-CC group = 9.

magnitude than were present during the control period.

DISCUSSION

The initial phase of this study was an attempt to demonstrate the transmission of "minor respiratory illnesses" (MRI) to human volunteers by the inoculation of filtered respiratory secretions obtained from 4 donor subjects whose illnesses appeared to differ in their clinical characteristics. Minor respiratory illnesses were induced by the inocula from all 4 of the donors and presumptive evidence was obtained for the existence of separate etiological "entities." Only 2 types of illness, however, could be distinguished. The first type, induced by the "acute respiratory disease" (ARD) filtrate, was characterized by prominence of pharyngeal involvement and an incubation period of 5 to 6 days. The second type, induced by both the "common cold" (CC) and "severe common cold" (S–CC) filtrates, was characterized chiefly by coryza and an incubation period of 24 to 48 hours. The illnesses induced by the inoculum from the fourth donor, whose infection was termed "bronchitis resembling atypical pneumonia" (Br–AP), were too few in number and variable in character to permit classification. The etiology and clinical nature of these induced minor respiratory illnesses will be discussed briefly in this paper. In the following paper, which presents the second phase of the study, the differentiation of these entities on an immunological basis will be considered and the relationship of the illnesses to primary atypical pneumonia will be discussed.

Consideration of the results of the initial experiments in terms of etiology led to the tentative con-

clusions that the illnesses induced in the volunteers were the direct results of inoculation of the various filtrates and were due to uncharacterized filtrable agents, presumably viruses, therein. Several types of data supported such conclusions: (*1*) Two distinct periods of "incubation" existed between the time of inoculation of 3 of the filtrates (ARD, S–CC and CC) and the onset of symptoms in the recipients. (*2*) Symptoms of respiratory disease did not follow the inoculation of a control group of 9 men with autogenous filtrates, thus excluding mechanical irritation and the excitation of latent agents as an explanation of the results. (*3*) There were no consistent alterations in the bacterial flora of the throat at any time, before or after inoculation, in the individuals of any group either with or without illness. The development of streptococcal antistreptolysins was not demonstrable in convalescent phase sera. (*4*) Serological tests excluded the influenza viruses A and B as causative agents. The recognized clinical variability of influenza and its experimental incubation period of 6 to 48 hours (27 to 30) made these tests necessary. Heterophile agglutinins could not be demonstrated. Finally, (*5*) the results of these experiments differed in 2 respects from those in which primary atypical pneumonia was transmitted (18), namely, (*a*) pulmonary infiltration was not demonstrable roentgenographically in any patient and (*b*) the incubation periods were shorter. On these bases, therefore, it seemed reasonable to consider the 2 types of illness induced in the volunteers as separate and distinct virus diseases.

The nature of the infections resulting from the administration of S–CC and CC inocula suggested that a virus similar to, if not identical with, that of the "common cold" virus was operative. Previous transmission experiments in volunteers (31 to 35) and apes (33, 36, 37) have demonstrated a coryza-like illness of short incubation period (8 to 70 hours) following the inoculation of filtered respiratory tract washings obtained from individuals with the "common cold." The data obtained in the present study confirm and extend these observations.

The evidence derived from the administration of ARD inoculum suggested that this filtrate contained an agent which was distinct from that of the "common cold" virus. The sharp difference in incubation period and, clinically, the paucity of nasal symptoms and the prominence of throat symptoms early in the course of disease, were the chief differential features. While such results have not been noted in previous transmission experiments (33, 34), it is of interest that a clinical and epidemiological study of acute minor respiratory diseases by Van Volkenburgh and Frost (8) suggested a "real difference" between cases of "sore throat" and those of "coryza" and "cough," which was "in accordance with the prevailing view that a considerable proportion of the cases of sore throat ordinarily encountered are not related to the common cold."

The small number of infections with variable incubation periods resulting from the inoculation of Br–AP filtrate permitted no conclusions regarding the nature of the agent present in this inoculum. It was anticipated that this group of volunteers would develop moderately severe respiratory illnesses with a long incubation period (approximately 2 weeks), since the donor of the inoculum had a more severe form of respiratory disease which resembled primary atypical pneumonia. None of the recipients, however, developed an infection of comparable degree.

A feature of considerable interest in this study was the mild nature of the illnesses which followed the several types of inoculum. Objective signs of illness, such as fever, or constitutional symptoms were not uniformly observed in patients of the ARD, S–CC and Br–AP groups, as had been noted in the donors.

The explanation for the mildness of the illness is unknown. It seems possible that a combination of circumstances was responsible. On the basis of a history of previous respiratory disease, these individuals had apparently been susceptible to respiratory infections in their ordinary walks of life and presumably were susceptible at the time of inoculation. Several factors, however, may have been operative to increase their resistance. Although the men were isolated and restricted in activity, the living conditions were those generally considered to be favorable for good health, namely, adequate rest, good nutrition, absence of physical fatigue and over-exertion, etc. The investigations were purposely conducted during the summer months when respiratory infections in the general population are usually infrequent and uncompli-

cated. The environmental temperature was relatively constant, so that the potentially adverse effects of dermal chilling (38, 39) and of meteorological changes (40, 41) were avoided. The factor of dosage may have been of importance, but cannot be evaluated. Thus the possibility exists that some of the illnesses would have been more severe had the conditions of the study permitted more variation in the environmental and host factors.

SUMMARY

Minor respiratory illnesses were transmitted to human volunteers by the use of bacteria-free filtrates of pooled nasal and pharyngeal washings obtained from 4 single donors. Two of the donors had experienced coryza-like illnesses (S–CC and CC), 1 had had an infection with sore throat as a principal symptom (ARD), and the fourth suffered from a more severe form of illness (Br–AP) which clinically resembled primary atypical pneumonia. As a control group, 9 single donors were inoculated with autogenous filtered washings.

Two types of illness were induced; one was a coryzal-like infection developing after an interval of 24 to 48 hours and the other, an infection characterized by prominence of sore throat, minimal nasal symptoms and an incubation period of 5 to 6 days. These illnesses were clinically distinguishable. The control group which received autogenous filtrates experienced no illnesses. Primary atypical pneumonia did not develop in any of the subjects.

The results of these experiments indicate that at least 2 filtrable agents, presumably viruses, may induce minor respiratory illness in man.

BIBLIOGRAPHY

1. Dingle, J. H., Abernethy, T. J., Badger, G. F., Buddingh, G. J., Feller, A. E., Langmuir, A. D., Ruegsegger, J. M., and Wood, W. B., Jr., Primary atypical pneumona, etiology unknown. Am. J. Hyg., 1944, 39, 67.
2. Commission on Acute Respiratory Diseases, Epidemiology of atypical pneumonia and acute respiratory disease at Fort Bragg, North Carolina. Am. J. Pub. Health, 1944, 34, 335.
3. Bock, A. V., Clinical observations, complications, and treatment of acute upper respiratory tract infections. Ann. Int. Med., 1938, 12, 317.
4. The School Epidemics Committee, Epidemics in schools; an analysis of the data collected during the first five years of a statistical inquiry. Medical Research Council, Special Reports Series, No. 227, 1938, London, His Majesty's Stationery Office.
5. Murray, M. E., Atypical bronchopneumonia of unknown etiology possibly due to a filtrable virus. New England J. Med., 1940, 222, 565.
6. Reimann, H. A., and Havens, W. P., An epidemic disease of the respiratory tract. Arch. Int. Med., 1940, 65, 138.
7. Frost, W. H., and Gover, M., The incidence and time distribution of common colds in several groups kept under continuous observation. Pub. Health Rep., 1932, 47, 1815.
8. Van Volkenburgh, V. A., and Frost, W. H., Acute minor respiratory diseases prevailing in a group of families residing in Baltimore, Maryland, 1928–1930. Prevalence, distribution and clinical description of observed cases. Am. J. Hyg., 1933, 17, 122.
9. Frost, W. H., and Van Volkenburgh, V. A., The minor respiratory diseases as observed during the influenza epidemic of 1928–29 and in a nonepidemic period. Am. J. Hyg., 1935, 21, 647.
10. Hare, R., Hamilton, J., and Feasby, W. R., Influenza and similar respiratory infections in a military camp over a period of 3 years. Canad. J. Pub. Health, 1943, 34, 453.
11. Kornblum, K., and Reimann, H. A., The roentgenological aspects of an epidemic of acute respiratory tract infection. Am. J. Roentgenol., 1940, 44, 333.
12. Commission on Acute Respiratory Diseases, Endemic exudative pharyngitis and tonsillitis: etiology and clinical characteristics. J. A. M. A., 1944, 125, 1163.
13. Commission on Acute Respiratory Diseases and Commission on Air-Borne Infections. A study of the effect of oiled floors and bedding on the incidence of respiratory disease in new recruits. Am. J. Hyg., 1946, 43, 120.
14. Commission on Acute Respiratory Diseases. Clinical patterns of undifferentiated and other acute respiratory diseases in Army recruits. Unpublished data.
15. Commission on Acute Respiratory Diseases, Acute respiratory disease among new recruits. Am. J. Pub. Health, 1946, 36, 439.
16. Commission on Acute Respiratory Diseases, The present status of the etiology of primary atypical pneumonia. Bull. N. Y. Acad. Med., 1945, 21, 235.
17. Commission on Acute Respiratory Diseases, An experimental attempt to transmit primary atypical pneumonia in human volunteers. J. Clin. Invest., 1945, 24, 175.
18. Commission on Acute Respiratory Diseases, The transmission of primary atypical pneumonia to human volunteers: I. Experimental methods; II. Results of inoculation; III. Clinical features; IV. Laboratory studies. Bull. Johns Hopkins Hosp., 1946, 79, 97.

19. Rourke, M. D., and Ernstene, A. C., A method for correcting the erythrocyte sedimentation rate for variations in the cell volume percentage of blood. J. Clin. Invest., 1930, 8, 545.
20. Commission on Acute Respiratory Diseases, Cold hemagglutinins in primary atypical pneumonia and other respiratory infections. Am. J. M. Sc., 1944, 208, 742.
21. Hodge, B. E., and Swift, H. F., Varying hemolytic and constant combining capacity of streptolysins; influence on testing for antistreptolysins. J. Exper. Med., 1933, 58, 277.
22. Paul, J. R., and Bunnell, W. W., The presence of heterophile antibodies in infectious mononucleosis. Am. J. M. Sc., 1932, 183, 90.
23. Hirst, G. K., and Pickels, E. G., A method for the titration of influenza hemagglutinins and influenza antibodies with the aid of a photoelectric densitometer. J. Immunol., 1942, 45, 273.
24. Commission on Acute Respiratory Diseases, in collaboration with Mickle, W. A., Jr., and Oliver, T. J. Problems in determining the bacterial flora of the pharynx: I. Technique of examining a throat swab. Unpublished data.
25. Pike, R. M., An enrichment broth for isolating hemolytic streptococci from throat swabs. Proc. Soc. Exper. Biol. and Med., 1944, 57, 186.
26. Commission on Acute Respiratory Diseases, Experimental transmission of minor respiratory illness to human volunteers by filter-passing agents: II. Immunity on reinoculation with agents from two types of minor respiratory illness and from primary atypical pneumonia. J. Clin. Invest., 1947, 26, 974.
27. Smorodintseff, A. A., Tushinsky, M. D., Drobyshevskaya, A. I., Korovin, A. A., and Osetroff, A. I., Investigation on volunteers infected with the influenza virus. Am. J. M. Sc., 1937, 194, 159.
28. Henle, W., Henle, G., and Stokes, J., Jr., Demonstration of the efficacy of vaccination against influenza type A by experimental infection of human beings. J. Immunol., 1943, 46, 163.
29. Francis, T., Jr., Pearson, H. E., Salk, J. E., and Brown, P. N., Immunity in human subjects artificially infected with influenza virus, type B. Am. J. Pub. Health, 1944, 34, 317.
30. Francis, T., Jr., Salk, J. E., Pearson, H. E., and Brown, P. N., Protective effect of vaccination against induced influenza A. J. Clin. Invest., 1945, 24, 536.
31. Kruse, W., Die Erreger von Husten und Schnupfen. Muench. Med. Woch., 1914, 61, 1547.
32. Foster, G. B., Jr., The etiology of common colds; the probable role of a filtrable virus as the causative factor: with experiments on the cultivation of a minute micro-organism from the nasal secretion filtrates. J. Inf. Dis., 1917, 21, 451.
33. Dochez, A. R., Shibley, G. S., and Mills, K. C., Studies in the common cold. IV. Experimental transmission of the common cold to anthropoid apes and human beings by means of a filtrable agent. J. Exper. Med., 1930, 52, 701.
34. Long, P. H., Doull, J. A., Bourn, J. M., and McComb, F., The etiology of acute upper respiratory infection (common cold). J. Exper. Med., 1931, 53, 447.
35. Andrewes, C. H., and Oakley, W. G., The common cold wins the first round. St. Bartholomew's Hospital Journal, 1933, p. 74.
36. Dochez, A. R., Shibley, G. S., and Mills, K. C., A study of acute infections of the respiratory tract in the ape. Proc. Soc. Exper. Biol. and Med., 1929, 26, 562.
37. Shibley, G. S., Mills, K. C., and Dochez, A. R., Further consideration of the transmissibility of human upper respiratory infections (common cold) to the ape. Proc. Soc. Exper. Biol. and Med., 1929, 27, 59.
38. Hill, L., The science of ventilation and open-air treatment. Medical Research Council, Special Reports Series, No. 32, Part 1, 1919, London, His Majesty's Stationery Office.
39. Mudd, S., Grant, S. B., and Goldman, A., The etiology of acute inflammations of the nose, pharynx and tonsils. Ann. Otol. Rhinol. and Larnygol., 1921, 30, 1.
40. Van Loghem, J. J., An epidemiological contribution to the knowledge of respiratory diseases. J. Hyg., 1928, 28, 33.
41. Young, M., The influence of weather conditions on the mortality from bronchitis and pneumonia in children. J. Hyg., 1924, 23, 151.

EXPERIMENTAL TRANSMISSION OF MINOR RESPIRATORY ILLNESS TO HUMAN VOLUNTEERS BY FILTER-PASSING AGENTS. II. IMMUNITY ON REINOCULATION WITH AGENTS FROM THE TWO TYPES OF MINOR RESPIRATORY ILLNESS AND FROM PRIMARY ATYPICAL PNEUMONIA[1].

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INTRODUCTION

In the previous paper (1) it was shown that minor respiratory illnesses were induced in volunteers by means of bacteria-free filtrates obtained from representative cases of acute respiratory disease. Two distinct types of minor respiratory illness resulted: one, a coryza-like infection with a short incubation period of 1 to 2 days (filtrates S–CC and CC); the other, an infection developing after 5 to 6 days and characterized by prominence of sore throat and minimal nasal symptoms (filtrate ARD). A control group of volunteers given autogenous filtrates remained well. None of the subjects developed primary atypical pneumonia following the inoculation of any of these filtrates.

The present paper reports the results of reinoculation of some of the subjects with agents from the 2 types of minor respiratory illness and from primary atypical pneumonia. These investigations were undertaken in order to determine whether or not immunity followed inoculation with the agents. It was found that homologous immunity was present in individuals who had experienced the "sore throat," long-incubation-period disease (filtrate ARD); little or no immunity was demonstrated in those who had had coryza-like illness of short incubation (filtrate S–CC). Cross immunity with either filtrate was not found. Primary atypical pneumonia was induced subsequently in these volunteers who had had prior exposure to ARD and S–CC filtrates.

MATERIALS AND METHODS

Subjects. The volunteer group consisted of 41 healthy adult white males. Of this number, 16 had participated in the previous study; the remaining 25 were replacements.

Isolation procedures. Isolation was continued without interruption in the group of 16 men who remained for the second half of the study. Isolation precautions for the group of incoming men were instituted as soon as practicable after arrival. Partitions in the hallways on the third floor of the hotel separated 20 of the "new" volunteers into 2 groups of 10 men each. Similar partitions on the second floor divided the remaining 21 into groups of 11 and 10 men, respectively. One of these groups consisted of 6 men previously inoculated and 5 replacements; the other consisted of 10 men who had had previous inoculations.

Inocula. The inocula consisted of the same bacteria-free filtrates ARD, S–CC and CC which were utilized in the first half of the study. In addition, a filtrate of respiratory secretions from a patient with primary atypical pneumonia was employed. The illness in this donor began on May 9, 1945, and was characteristic of a moderately severe infection. Constitutional symptoms, pro-

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TABLE I

Titers of cold hemagglutinins in sera from donor of atypical pneumonia filtrate

Day of illness	Final dilution of serum			
	8	16	32	64
2	2*	±	0	0
9	4	1	1	0
15	2	0	0	0

* 0 = no visible agglutination.
± = doubtful agglutination.
1 to 4 = increasing degrees of agglutination.

ductive cough and substernal pain were prominent features. Pulmonary infiltration was present in the left and right lower lobes. The febrile course lasted 6 days. There was a slight rise and subsequent fall in the titer of cold hemagglutinins in 3' specimens of sera, examined simultaneously (Table I). Specimens of sputum and pharyngeal washings were collected during the febrile period, pooled, and a filtrate prepared. Aerobic and anaerobic cultures of filtrate were sterile.

Plan of study. Four experiments were performed over the course of approximately 7 weeks. Experiment I consisted of the reinoculation of 16 men who had participated in the previous study; 11 men were challenged with ARD or S–CC filtrate and 5 men, formerly inoculated with autogenous washings (Controls), were given filtrate S–CC. Experiment II consisted of the reinoculation of the above 11 men with heterologous filtrates, ARD and S–CC, respectively, and the inoculation of a control group of 5 "new" volunteers with filtrate ARD. Finally, the 21 men above were given filtrate obtained from a patient with primary atypical pneumonia (Experiment III). Additional studies were made in 2 groups of 10 men each who had formerly received filtrates CC and Br–AP (Experiment IV). These men were inoculated with coryza filtrate CC in order to test homologous immunity in one and heterologous immunity in the other.

Inoculation procedure. The technique of inoculation and the type of inoculation in general were the same as those employed in the first half of the study. When adequate amounts of filtrate were available the total inoculum was 10 ml.; this was given to each man in 3 equal doses on a single day. A reduction in total dosage to 6.6 ml. was necessary with one filtrate (ARD) and to 5.0 ml. in the case of another (S–CC). These were administered in 2 equal doses on a single day.

Clinical and laboratory studies. Each of the men was observed daily for the development of symptoms and signs of respiratory disease. The procedures employed have been described (1).

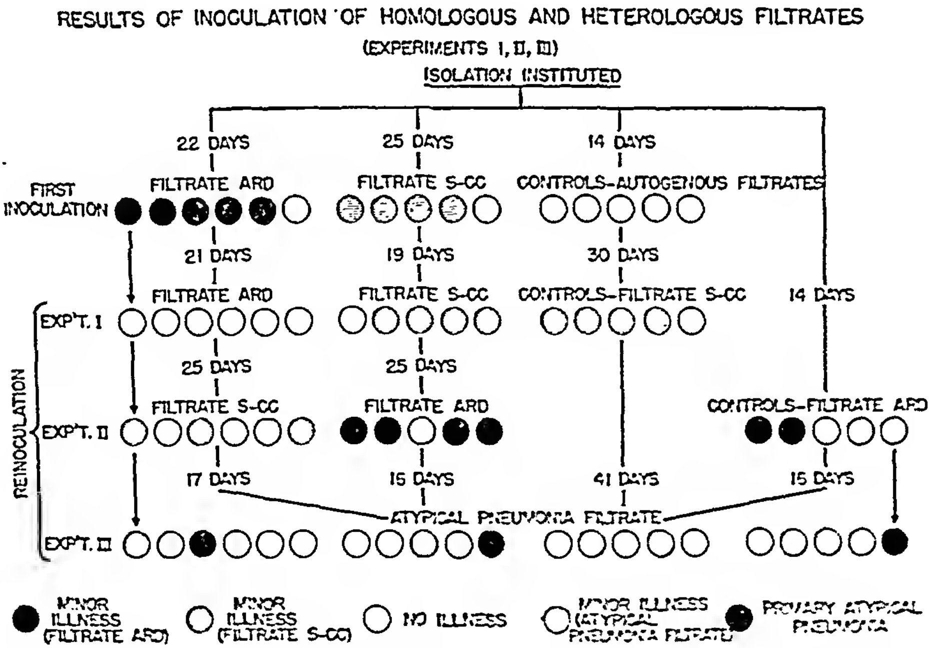

FIG. 1. THE RESULTS OF REINOCULATION OF HOMOLOGOUS (EXPERIMENT I) AND HETEROLOGOUS (EXPERIMENT II) FILTRATES AND OF CHALLENGE WITH ATYPICAL PNEUMONIA FILTRATE (EXPERIMENT III)

Each circle represents a volunteer and the varied shadings indicate the designated type of illness. The sequence of events for each man is indicated by a single vertical column of circles.

RESULTS

The effect of inoculation of homologous filtrate (Experiment I)

Approximately 3 weeks after initial inoculation with filtrates ARD and S–CC, 2 groups totaling 11 men, were challenged with homologous filtrates (Figure 1). Five of the 6 men originally exposed to ARD filtrate had experienced illnesses beginning 5 or 6 days after inoculation; 4 of 5 men given S–CC inoculum had developed characteristic coryzal illnesses after 1 to 2 days. At the same time a control group of 5 men, who had been inoculated 30 days previously with autogenous washings and had remained well, was given filtrate S–CC.

None of the 6 individuals receiving ARD filtrate a second time developed symptoms or signs of respiratory illness during the course of 25 days. In contrast, 4 of 5 men inoculated with homologous S–CC filtrate developed illnesses. Similarly, 4 of 5 men in the control group receiving S–CC filtrate developed illnesses.

The characteristics of the illnesses in these cases receiving S–CC filtrate were in general similar to those previously noted with this type of inoculum. Onset of symptoms occurred on the 1st or 2nd day in 7 of the 8 cases in both groups. Nasal symptoms and signs were the most characteristic features of the illnesses. In general, however, those who had experienced previous illness as a result of S–CC filtrate suffered from a somewhat milder infection upon reinoculation. This was evidenced principally by the short duration of symptoms which averaged 3 to 4 days.

Comment. The results of this experiment indicated immunity to homologous filtrate in subjects developing the "sore throat," long incubation period disease (ARD). Little or none was demonstrated in those experiencing coryza-like illnesses of short incubation (S–CC). The development of illness in the control group showed that filtrate S–CC, at least, was infectious in reduced dosage and still active after an additional month's storage. That filtrate ARD was also infectious at this time was indicated by a subsequent experiment, to be described below, in which illnesses were induced in another group of subjects.

The effect of inoculation of heterologous filtrate (Experiment II)

Twenty-five days after reinoculation of homologous filtrates ARD and S–CC, these 11 men were given heterologous filtrates (Figure 1). Six men, having received 2 inoculations with ARD filtrate, were given S–CC filtrate and 5 men, exposed twice to S–CC inoculum, were inoculated with ARD filtrate. A control group of 5 replacements also received ARD filtrate in order to test the activity of this material. The dosages employed were the same as in Experiment I; namely, 6.6 ml. of ARD filtrate and 5.0 ml. of S–CC filtrate.

As shown in Figure 1, 3 of 6 men given filtrate S–CC and 4 of 5 men inoculated with filtrate ARD developed respiratory illnesses. In the control group 2 of 5 men also became ill.

The types of illness occurring in this experiment were in general the same as those noted previously. The 3 individuals with illness who had been given filtrate S–CC developed onsets of disease on the 2nd and 3rd days after inoculation. Nasal symptoms and signs were prominent in each case. Fever, however, was not present. Those receiving filtrate ARD developed onsets as follows: 2nd day (1 case), 5th or 6th day (4 cases), 7th day (1 case). Symptoms and signs referable to the throat were characteristic. Three individuals displayed fever between the 8th and 10th days, varying between 99° and 100.8° F. One subject whose symptoms began on the 2nd day had fever beginning on the 4th day after inoculation. He was moderately ill over the course of 6 days, the maximum temperature reaching 100.8° F. Constitutional symptoms, sore throat, hoarseness, enlarged tender cervical lymph nodes and signs of pharyngeal inflammation were prominent features of illness.

Comment. The results of this experiment indicated a lack of immunity in subjects given heterologous filtrates ARD and S–CC. There was no indication that antecedent illness, brought about by one filtrate, modified the number or lessened the severity of illness which followed the administration of the heterologous material. Both types of infection, one with a short and the other with a long incubation period, were again induced in subjects even though the inoculum was reduced in amount and all of the individuals had at some

time previously experienced recent respiratory illness.

The effect of inoculation of filtrate from a case of primary atypical pneumonia (Experiment III)

The 21 volunteers who had participated in Experiments I and II were finally inoculated with filtrate obtained from a single donor early in the course of primary atypical pneumonia.

The experimental group was composed of the following: 6 men who had received filtrate ARD on 2 occasions and then filtrate S–CC; 5 men given filtrate S–CC twice and then filtrate ARD; and 2 groups of 5 men, each of whom had received filtrate ARD or S–CC on one occasion. The interval between the last inoculation was 16 or 17 days in 3 of the groups, and 41 days in the case of 1 of the former control groups. The total inoculum administered to each of the 21 men was 10 ml. No additional volunteers were available to serve as a control of this inoculum.

Three cases of atypical pneumonia and 1 of minor respiratory illness were observed (Figure 1). The remainder of the group, 17 individuals, failed to show evidence of respiratory infection. Two of the 3 cases of atypical pneumonia and the one instance of minor respiratory illness occurred in individuals who had experienced recent illnesses as a result of inoculation of both filtrates, ARD and S–CC. The remaining case, an individual with atypical pneumonia, had had no infection as a result of previous inoculation with filtrate ARD. No illnesses occurred in this experiment in any of the 5 men originally given autogenous washings and subsequently exposed to filtrate S–CC.

The 3 individuals with pneumonia had illnesses characteristic not only of the naturally acquired infection (2), but also of the experimentally induced disease (3). Onset of symptoms occurred on the 13th, 14th, and 15th days after inoculation, respectively. The illnesses were of mild severity; fever of 5 to 7 days' duration was present in each case. The maximum temperature in 1 individual was 102.4° F. Unilateral infiltration of one or the other lower lobes was observed in each case. No complications were noted; none of these patients developed cold hemagglutinins in their sera.

The patient with minor respiratory illness first developed symptoms on the 10th day after inoculation. His illness began with sore throat and slight nasal discharge. On the 15th day, headache, malaise, coryza, cough and substernal chest pain were noted. Physical examination revealed no significant abnormalities. Pulmonary infiltration was not detected. Significant fever was not present. The duration of symptoms was 9 days. The patient described this illness as a "chest cold" in contrast to the "head cold" he had experienced following the last inoculation (filtrate S–CC).

Comment. The results of this experiment demonstrate that previous minor respiratory illness did *not* give immunity to primary atypical pneumonia.

These results confirm previous observations that primary atypical pneumonia may be induced in volunteers by means of bacteria-free filtrates (3). In addition, they demonstrate that transmission of the disease may be accomplished with a filtrate obtained from a single donor as well as with the pooled filtrates from several donors. In previous studies, however, it was noted that approximately half of the volunteers developed minor illnesses after inoculation with pooled atypical pneumonia filtrate. Only 1 such illness developed in this experiment.

Additional studies on homologous and heterologous immunity (Experiment IV)

For further studies on the effect of reinoculation of CC filtrate, 20 additional volunteers were available. One group of 10 men had been inoculated with "coryza" filtrate CC; the other group of similar size had received filtrate Br–AP from a patient with respiratory disease termed "bronchitis resembling atypical pneumona" (2). The results of inoculation in the former group had shown 6 coryza-like illnesses with an incubation period of 1 to 4 days. Following the latter inoculation 4 illnesses of ill-defined nature had developed.

Approximately 3 weeks later both groups were reinoculated with 10 ml. of "coryza" filtrate CC. Thus, homologous immunity in one group and heterologous immunity in the other, were tested. The results are shown in Figure 2.

Four minor respiratory illnesses developed in men given the heterologous material and 3 in those who were challenged with the same filtrate (CC). Onset of symptoms in these 7 cases occurred on

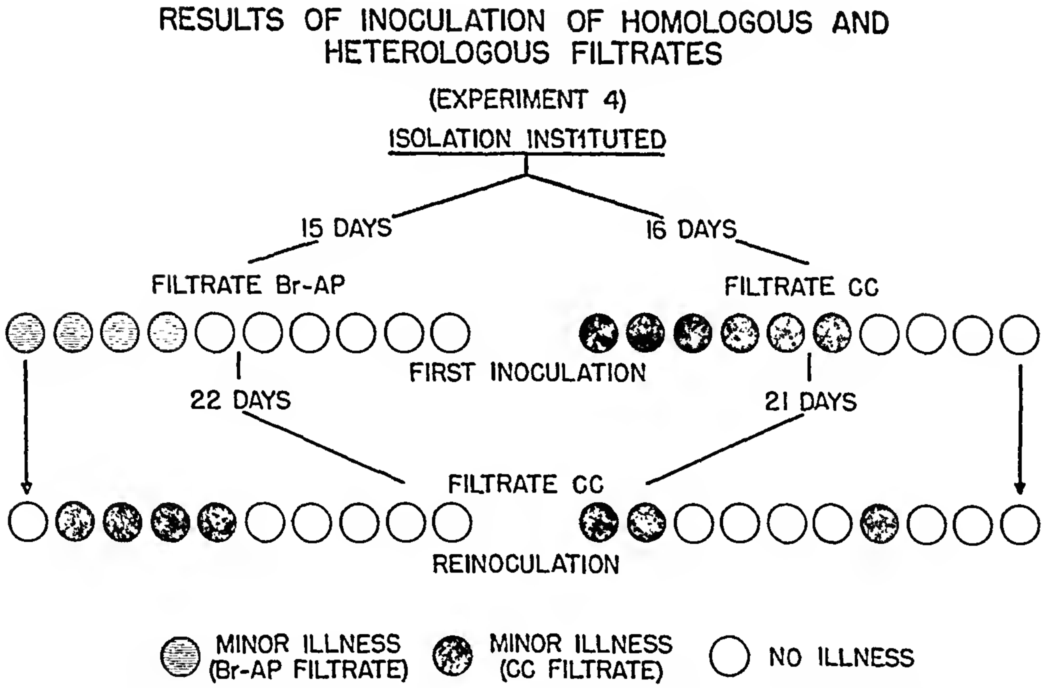

FIG. 2. THE RESULTS OF REINOCULATION, WITH "COMMON COLD" FILTRATE (CC), OF 2 GROUPS PREVIOUSLY EXPOSED EITHER TO THE HOMOLOGOUS FILTRATE (CC) OR TO HETEROLOGUS FILTRATE FROM A CASE TERMED "BRONCHITIS RESEMBLING ATYPICAL PNEUMONIA" (Br–AP)

the 1st and 2nd day in 5 cases and on the 9th day in 2 cases. Nasal symptoms and signs of variable intensity were found in all but 1 case. Cough was noted by 3 patients. Other symptoms noted infrequently were headache, malaise, sore throat and chest pain. The illnesses were afebrile and of 2 to 5 days' duration.

Comment. These results confirm the findings in Experiments I and II to a limited extent. Employing 1 type of "coryza" filtrate CC, subjects challenged with the same filtrate again developed characteristic illnesses. Other subjects previously inoculated with filtrate Br–AP developed illnesses when exposed to heterologous filtrate CC. Thus, little or no evidence of cross immunity was demonstrated. The explanation for the delayed incubation period of 9 days in 2 cases was not apparent.

Laboratory studies. Examination of the throat flora at 2-day intervals in all subjects failed to show any significant change as result of inoculation in the frequency of the pneumococcus, *Beta*-hemolytic streptococci, *Staphylococcus aureus, Hemophilus influenzae, H. hemolyticus, Klebsiella pneumoniae* and gram-negative cocci. Furthermore, the distribution of specific types of pneumococci and of *Beta*-hemolytic streptococci in volunteers, attendants and professional staff revealed no evidence of spread during the course of these experiments.

The total and differential leucocyte counts were within normal limits in all volunteers. No consistent alterations were evident in either type of examination as a result of any of the various types of inoculations. Similarly, the corrected erythrocyte sedimentation rates were normal in all subjects.

A rise in titer of cold hemagglutinins (4-fold increment) was found in the serum of but 1 individual (Table II). This volunteer, during the pre-inoculation control period, showed a cold hemagglutinin titer of 16. Following the administration of autogenous washings and of filtrate S–CC

TABLE II

*Results of cold hemagglutinin tests**

Experiment	Number of observations	Rise in cold hemagglutinin titer		
		4-fold	<4-fold	No rise
I	16	0	1	15
II	16	0	1	15
III	21	1	3	17
IV	20	0	1	19

* Based on weekly samples of serum obtained throughout the period of observation. Titer of the last serum specimen before each inoculation was considered the pre-inoculation titer.

the *maximum* titer on repeated occasions was 8. A titer of 32 was noted in the serum 14 days after inoculation of atypical pneumonia filtrate. He experienced no illness with the latter filtrate.

Appropriate sera from each individual were tested for antibodies against influenza viruses A and B and also streptolysin "O." In no instance was a diagnostic increase in titer observed.

DISCUSSION

The studies in human volunteers presented in this report and in the preceding paper (1) constituted an attempt to segregate "entities" in the group of unclassified respiratory diseases and to define more clearly the relation between minor respiratory illness and primary atypical pneumonia. These objectives were partially achieved. Two types of minor respiratory illness were induced which appeared to be distinct entities having no demonstrable immunological relationship. The inocula giving these 2 types of minor illness did not induce primary atypical pneumonia, nor did they prevent the subsequent development of primary atypical pneumonia. It would thus appear that 3 separate entities have been dealt with in these experiments. Limitations of the data, however, prohibit the conclusion that the agent of primary atypical pneumonia is not associated with minor respiratory illness. The results will be considered further under the following headings: Minor respiratory illness induced by the ARD filtrate, minor respiratory illness induced by the CC filtrate, and the relation of minor respiratory illness and primary atypical pneumonia.

Minor respiratory illness induced by the ARD filtrate. Minor respiratory illness, characterized predominantly by sore throat and an incubation period of from 5 to 6 days, was induced with the ARD filtrate. The inoculation of the same filtrate 21 days later demonstrated an apparently solid immunity. This conclusion appears to be valid since the challenge filtrate produced illness in a control group of "new" men inoculated at a later date; and because there seems to be no reason to assume the existence of a refractory state, such as that observed in the ferret by Stuart-Harris and Francis (4), or the operation of the interference phenomenon (5). Moreover, these subjects were susceptible to S–CC filtrate 25 days after they had been challenged with the ARD filtrate (46 days after their initial inoculation).

The minor respiratory illnesses induced by the ARD filtrate, after an incubation of 5 to 6 days, appear to constitute a new entity which has not been described previously in transmission experiments. It seems possible that this entity, despite its mild nature in the recipients (1), is the same as that observed in recruits and termed "undifferentiated acute respiratory disease" (ARD). Clinical studies of these illnesses in recruits have shown them to be primarily noncoryzal respiratory infections (6). The illnesses likewise may be similar to those termed cases of "sore throat" by Van Volkenburgh and Frost (7) in their studies in a civilian population. Epidemiological observations have not defined the incubation period but have suggested the development of immunity as indicated by the absence of epidemics of ARD in seasoned troops as contrasted with recruits (8). Finally, the washings used in these experiments were obtained from a recruit whose illness was considered clinically to be typical of ARD.

Minor respiratory illness induced by CC filtrates. The minor respiratory illnesses induced by filtered washings from 2 cases, referred to here as "common cold," were characterized by predominance of coryza and an incubation period of 1 to 2 days. No appreciable homologous immunity could be demonstrated in the S–CC and CC groups following inoculation with the respective filtrates at intervals of 19 days (S–CC) and 21 days (CC). Heterologous immunity to the ARD filtrate was not demonstrated. The control experiments, in which autogenous filtrates were employed, indicate that these results were not due to mechanical irritation of the respiratory passages or the result of stimulation of a latent agent already present in the respiratory tract.

Minor respiratory illnesses produced by these CC filtrates appear to be similar to, if not identical with, the illnesses transmitted by Kruse (9), Foster (10), Dochez *et al* (11), and Long *et al* (12), and termed by them the "common cold." The failure to demonstrate immunity to the CC filtrate in the present experiments is contrary to the opinion generally held that such minor infections have an immunity of approximately 3 or 4 months. They

are in accord, however, with findings in the Abel Fund Studies (13). Previous investigators did not attempt transmission experiments during the 3 or 4 months following a cold or an experimentally induced infection.

The frequency with which this minor respiratory illness, which resembles the "common cold," occurs in recruits and seasoned troops has not been adequately determined. It is possible, however, that this disease is similar to the minor illnesses observed in field surveys and in dispensaries during the summer and fall months.

Relation of minor respiratory illness and primary atypical pneumonia. Clinical and epidemiological studies of sporadic and epidemic primary atypical pneumonia have shown, in many instances, an apparent association between this disease and minor respiratory illnesses (2, 3, 14 to 17). These findings have led to the hypothesis that the same agent produces both types of infection and that primary atypical pneumonia may actually be an infrequent manifestation of pulmonary involvement in a prevalent minor respiratory disease. Such a theory seems reasonable and is not novel, since it is in accord with the behavior of such known agents as influenza virus A, the meningococcus, etc. This interpretation may likewise be placed on the demonstration, in 1944, that minor respiratory illnesses and primary atypical pneumonia were induced in 50 per cent and 25 per cent, respectively, of a group of volunteers inoculated with pooled filtered secretions of the respiratory tracts of 6 patients with primary atypical pneumonia (3).

The above evidence alone is not sufficient to prove the hypothesis, however, because the presence of only one agent cannot be established. Moreover, minor respiratory illnesses have been infrequent or absent in some outbreaks of primary atypical pneumonia (18, 19) and have varied in their relative sporadic frequency (20). It is therefore possible that more than 1 agent may cause both sporadic and epidemic primary atypical pneumonia and minor respiratory illness. In support of this assumption may be the occurrence of cold hemagglutinins in some but not all cases of primary atypical pneumonia (21–24).

In the present study, further evidence was sought in 2 ways, (*a*) by an attempt to produce primary atypical pneumonia with washings from each of 2 cases (ARD and Br–AP) considered clinically and epidemiologically to be most closely related to that disease, as well as from 2 unrelated cases (S–CC and CC); and (*b*) by an attempt to demonstrate the presence or absence of immunity to primary atypical pneumonia in individuals who had previously been infected with ARD and S–CC filtrates.

Primary atypical pneumonia was not induced by the administration of inocula from any of the 4 cases, nor did previous inoculation with the ARD and S–CC filtrates prevent the subsequent development of the disease. Moreover, the incubation periods of the minor respiratory illnesses which did follow the initial inoculations—approximately 5 to 6 days with the ARD filtrate and approximately 1 to 3 days with the S–CC, CC and Br–AP filtrates—were much shorter than that found with the AP filtrates; that is, approximately 14 days (3).

It is difficult to interpret the occurrence of only one case of minor respiratory illness following the challenge inoculation of filtrate from a single case of primary atypical pneumonia (Figure 1) because previously uninoculated control subjects were not available. The absence of minor respiratory illnesses was in definite contrast to the results obtained in 1944 when pooled filtrates from 6 donors were employed (3). Theoretical explanations based on the assumption of the presence of 2 agents in the 1944 filtrates, or partial immunity induced by the ARD and S–CC inocula, do not appear to be adequate because of the absence of mild illnesses in the S–CC control group (Figure 1). One possibility appears to be that the agents producing primary atypical pneumonia in the 1944 and 1945 experiments were different. In support of this assumption may be the consistent occurrence of cold hemagglutinins in the experimental cases of primary atypical pneumonia in 1944 and their absence in the 3 cases induced in the present study. Further investigations of this aspect of the problem are needed before a final conclusion can be reached.

SUMMARY

Experiments were undertaken in order to determine the effects of reinoculation of bacteria-free filtrates obtained from representative donors

with acute respiratory disease. Homologous and heterologous immunity was tested. In addition, immunity to primary atypical pneumonia was assessed in subjects who were previously exposed to these filtrates and who contracted minor respiratory illness.

Immunity to reinoculation was found in individuals receiving filtrate ARD which induced minor illness of relatively long incubation period (5 to 6 days). Little or none was found in subjects challenged with filtrate S–CC inducing a coryza-like illness of short incubation (1 to 2 days). Cross-immunity was not demonstrable with either filtrate.

Primary atypical pneumonia developed in 3 volunteers following the inoculation of filtrate from a single donor who had atypical pneumonia. Each of these 3 patients had had 1 or more previous inoculations with ARD or S–CC filtrates; 2 had experienced recent minor respiratory illnesses.

The results of this study provide additional proof that at least 2 filtrable agents, or viruses, may induce minor respiratory illness in man and suggest that these agents are probably distinct from the virus or viruses of primary atypical pneumonia.

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BIBLIOGRAPHY

1. Commission on Acute Respiratory Diseases, Experimental transmission of minor respiratory illness to human volunteers by filter-passing agents. I. Demonstration of two types of illness characterized by long and short incubation periods and different clinical features. J. Clin. Invest., *1947, 26, 957.*
2. Dingle, J. H., Abernethy, T. J., Badger, G. F., Buddingh, G. J., Feller, A. E., Langmuir, A. D., Ruegsegger, J. M., and Wood, W. B., Jr., Primary atypical pneumonia, etiology unknown. Am. J. Hyg., 1944, 39, 67.
3. Commission on Acute Respiratory Diseases, The transmission of primary atypical pneumonia to human volunteers. I. Experimental methods, II. Results of inoculation, III. Clinical features, IV. Laboratory studies. Bull. Johns Hopkins Hosp., 1946, 79, 97.
4. Stuart-Harris, C. H., and Francis, T., Jr., Studies on the nasal histology of epidemic influenza virus infection in the ferret. II. The resistance of regenerating respiratory epithelium to reinfection and to physicochemical injury. J. Exper. Med., 1938, 68, 803.
5. Rivers, T. M., Immunological and serological phenomena of virus diseases. Lane Medical Lectures: Viruses and Virus Diseases, 1939, Stanford University, California, Stanford University Press.
6. Commission on Acute Respiratory Diseases. Clinical patterns of undifferentiated and other acute respiratory diseases in Army recruits. Unpublished data.
7. Van Volkenburgh, V. A., and Frost, W. H., Acute minor respiratory diseases prevailing in a group of families residing in Baltimore, Maryland, 1928–1930: prevalence, distribution and clinical description of observed cases. Am. J. Hyg., 1933, 17, 122.
8. Commission on Acute Respiratory Diseases, Acute respiratory diseases among new recruits. Am. J. Pub. Health, 1946, 36, 439.
9. Kruse, W., Die Erreger von Husten und Schnupfen. München. med. Wchnschr., 1914, 61, 1547.
10. Foster, G. B., Jr., The etiology of common colds: the probable role of a filtrable virus as the causative factor: with experiments on the cultivation of a minute micro-organism from the nasal secretion filtrates. J. Infect. Dis., 1917, 21, 451.
11. Dochez, A. R., Shibley, G. S., and Mills, K. C., Studies in the common cold. IV. Experimental transmission of the common cold to anthropoid apes and

human beings by means of a filtrable agent. J. Exper. Med., 1930, 52, 701.

12. Long, P. H., Doull, J. A., Bourn, J. M., and McComb, F., The etiology of acute upper respiratory infection (common cold). J. Exper. Med., 1931, 53, 447.
13. Reed, L. J., Statistical treatment of the intervals between successive attacks of the common cold. Presented before the American Epidemiological Society, New York, N. Y., April 27, 1934 (Unpublished).
14. Reimann, H. A., and Havens, W. P., An epidemic disease of the respiratory tract. Arch. Int. Med., 1940, 65, 138.
15. Gallagher, J. R., Acute pneumonitis; report of 87 cases among adolescents. Yale J. Biol. and Med., 1941, 13, 663.
16. Iverson, H. A., An epidemic of acute respiratory disease associated with atypical pneumonia. Bull. Johns Hopkins Hosp., 1943, 72, 89.
17. Breslow, L., Epidemic of acute respiratory disease associated with atypical pneumonia. J. Clin. Invest., 1945, 24, 775.
18. Daniels, W. B., Bronchopneumonia of unknown etiology in a girls' school. Am. J. M. Sc., 1942, 203, 263.
19. Young, L. E., Storey, M., and Redmond, A. J., Clinical and epidemiologic features of an outbreak of primary atypical pneumonia of unknown etiology among hospital and medical school personnel. Am. J. M. Sc., 1943, 206, 756.
20. Commission on Acute Respiratory Diseases, Epidemiology of atypical pneumonia and acute respiratory disease at Fort Bragg, North Carolina. Am. J. Pub. Health, 1944, 34, 335.
21. Horstmann, D. M., and Tatlock, H., Cold agglutinins: a diagnostic aid in certain types of primary atypical pneumonia. J. A. M. A., 1943, 122, 369.
22. Commission on Acute Respiratory Diseases, Cold hemagglutinins in primary atypical pneumonia and other respiratory infections. Am. J. M. Sc., 1944, 208, 742.
23. Curnen, E. C., Mirick, G. S., Ziegler, J. E., Jr., Thomas, L., and Horsfall, F. L., Jr., Studies on primary atypical pneumonia. I. Clinical features and results of laboratory investigations. J. Clin. Invest., 1945, 24, 209.
24. Finland, M., Peterson, O. L., Allen, H. E., Samper, B. A., and Barnes, M. W., Cold agglutinins. II. Cold isohemagglutinins in primary atypical pneumonia of unknown etiology with a note on the occurrence of hemolytic anemia in these cases. J. Clin. Invest., 1945, 24, 458.

THE ESTIMATION OF SERUM SODIUM FROM BICARBONATE PLUS CHLORIDE [1]

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Since the body fluids are nearly neutral and since they contain no appreciable quantities of organic bases, the concentration of cations in these fluids must be equal to the concentration of the inorganic cations found in them, [Na] + [K] + [Ca] + [Mg]. This, in turn, must be equal to the total concentration of anions. The distribution of water between cells and extracellular fluids in the body appears to be governed mainly by the concentration in the extracellular fluids of sodium, the principal one of those osmotically active components which are restrained from crossing the cellular membranes. In addition, sodium constitutes the chief cation of the extracellular fluids. In normal serum it makes up more than 90 per cent of the total concentration of cations. Although the concentrations of the other cations profoundly influence physiological processes, they are so small and vary so little that their effects on osmotic and cation-anion equilibria are trifling. The concentration of sodium is, therefore, the best single criterion of the electrolyte osmotic pressure and the concentration of cations in the serum and extracellular fluids. The measurement of sodium has, however, been so laborious and time-consuming that it has been less useful for clinical than for investigative purposes.

In lieu of direct measurement the concentration of sodium has usually been estimated from the combined concentrations of bicarbonate and chloride. In normal subjects these 2 ions together make up from 85 to 90 per cent of the anions of serum, falling only a little short of equaling sodium. The remainder of the anion column is composed chiefly of protein, with an inconsiderable amount of inorganic phosphate, an almost negligible quantity of sulfate and a small and variable contribution of organic acids. While important variations of the concentrations of cations manifest themselves consistently in the concentration of sodium, variations of the anions are not so consistently evidenced in the concentrations of HCO_3 + Cl. If some other foreign or endogenous anion accumulates in the serum it will displace bicarbonate. In this case [Na] will exceed [HCO_3] + [Cl] by more than the usual quantity and can be estimated only by the aid of inference.

The object of this paper is to examine the accuracy of such inferences. With the flame photometer it will become possible to measure the sodium of serum with sufficient speed and facility to make this measurement clinically available. It is therefore relevant to ascertain the indications for such measurements and the advantages and disadvantages they have in comparison with measurements of [HCO_3] + [Cl] and other combinations of procedures. For routine purposes some selection is desirable for economy of time, facilities, and blood.

MATERIAL

The material on which this analysis is based consists of 205 measurements of sodium, chloride, and CO_2 of serum from 28 normal adults and 63 patients with a variety of diseases, made for various reasons during the years 1932 to 1947. In many instances other analyses were made. The subjects for clinical observation were not selected at random, but usually because some abnormality of sodium or of the relation between sodium and chloride + bicarbonate was suspected.

All normals and most patients were studied in the postabsorptive state before breakfast. Exceptions to this rule were made in emergencies; but in such instances the patients were almost invariably fasting.

METHODS

Blood was drawn and serum was separated and preserved with anaerobic precautions. Chloride was measured by Eisenman's adaptation of the method of Austin and Van Slyke (1) or by Hald's modification of Patterson's micromethod (1); carbon dioxide was measured by the technique of Van Slyke and Neill (1), with the constant volume apparatus. Analyses for sodium were done by the gravimetric method of Hald (2) or by means of the flame photometer (3), or both.

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CALCULATIONS

Values for sodium, chloride, bicarbonate and other electrolytic components are given in terms of combining equivalents. Actually the figures for bicarbonate do not represent bicarbonate, since the CO_2 tension of the blood was not determined. They represent total CO_2 minus the CO_2 that would be held in solution and as H_2CO_3 in the serum at 37.5° C. and a pH of 7.35.[2] Since the clinical

[2] For this calculation the value of 6.10 was used for pK_1'; (4).

TABLE I

Variations of serum electrolyte concentrations in health and disease

Number of Subjects	Number of Observations		$[HCO_3]$	[Cl]	Σ	[Na]	Δ Observed	Δ >11	Δ >16
			meq. per liter						
			Normal males*						
16	19	Maximum	30.5	104.4	132.7	143.3	10.9		
		Minimum	25.7	100.0	128.3	132.4	2.8		
		Mean	27.7	102.7	130.2	135.7	5.8		
		s.d. ±	1.34	1.13	1.58	2.28	2.26		
			Normal females†						
12	16	Maximum	28.1	106.8	133.2	136.7	8.4		
		Minimum	23.1	103.2	128.0	129.0	−1.3		
		Mean	25.2	105.3	130.5	133.4	4.0		
		s.d. ±	1.42	0.92	1.06	3.11	2.33		
			Addison's disease						
5	37	Maximum	27.5	107.1	131.5	141.0	18.2		
		Minimum	14.8	82.6	102.6	115.9	2.8	9	1
			Pulmonary disease						
6	18	Maximum	56.0	105.3	131.1	146.0	20.1		
		Minimum	19.8	75.1	112.4	122.8	5.7	4	2
			Diabetes mellitus						
18	45	Maximum	31.0	122.4	135.8	151.6	45.5		
		Minimum	3.6	87.1	99.3	119.5	1.8	34	21
			Renal disease						
13	23	Maximum	28.1	125.5	132.2	148.5	34.7		
		Minimum	4.9	81.3	97.5	116.2	0.7	16	12
			Mercury poisoning						
4	18	Maximum	24.3	131.8	152.4	167.6	23.4		
		Minimum	9.5	86.7	100.0	117.3	0.0	14	2
			Miscellaneous diseases						
17	29	Maximum	40.9	103.5	130.0	141.0	27.3		
		Minimum	9.7	67.8	91.6	116.0	0.1	20	10

* One subject was omitted because his [Na] and Δ differed consistently from the mean by more than 3 times the standard deviation.

† One subject was omitted because her [Cl] and Σ differed consistently from the mean by more than 3 times the standard deviation.

material was extremely heterogeneous pH must have varied widely. If it be assumed that it varied from 7.0 to 7.8, the error of assuming a pH of 7.35 would amount to ± 7 per cent of the bicarbonate value. In a serum with a normal bicarbonate concentration of 27 milliequivalents per liter this would be about 2 milliequivalents.

In the text and tables concentrations of Na, Cl and HCO_3, in meq. per liter, are represented with brackets, [Na], [Cl], [HCO_3]. The sum, [HCO_3] + [Cl], is represented by the symbol Σ, the difference, [Na] − ([HCO_3] + [Cl]), by the Symbol Δ.

RESULTS

In Table I the data are presented in summary to indicate the degree of variability of each function in normal individuals and in various types of disease. There is, as has been noted before (4, 5), a statistically significant difference between normal males and females with respect to both [HCO_3] and [Cl]. The former is about 2.5 meq. per liter lower, while [Cl] is about 2.5 meq. higher in females. Because these differences are reciprocally related, Σ is the same for the 2 sexes. In this series the mean [Na] is 2.5 meq. higher in males, but this is not statistically significant. One male and one female, though presumably normal, were omitted from the statistical calculations because their electrolytes departed in certain particulars widely from the means. If they had been included the means would have been only slightly altered, while the differences between the sexes would have been exaggerated. In each sex [Cl] varies less than any other function; but, because of the difference between the sexes, varies more than Σ does in males and females together. Relatively [HCO_3] varies most, but in actual milliequivalents variations of [Na] are almost twice as great. Beyond the reciprocal relation between [HCO_3] and [Cl] in the 2 sexes, no significant correlation can be found between any 2 functions in either or both sexes. Even [HCO_3] and [Cl] bear no significant relation to one another in either males or females. The cause of the variations of Δ is, therefore, not clear from these data. The mean value of Δ in these normal subjects is about 5 meq. per liter, with all but 2 of the observations falling between 0 and 8.5. In normal persons under standard postabsorptive conditions, therefore, [Na] can be predicted from Σ with an error that seldom exceeds 5 meq. per liter.

In pathological conditions this prediction is far less accurate. In 170 observations Δ exceeded 11 meq., its maximum value in normal subjects, 97 times. In 47 instances, or 28 per cent of the observations in disease, Δ exceeded 16 meq. per liter. These figures have no statistical significance since the data were obtained from patients selected, in the main, because some abnormality of serum electrolytes was suspected. They will serve the present purpose, however, because it is in just such cases that knowledge of [Na] is desirable. If it is assumed that the mean value of [Na] is Σ + 5 meq., the concentration of [Na] would have been underestimated by more than 10 meq. in 28 per cent of the pathological subjects of this series.

DISCUSSION

It remains to examine the causes of large values of Δ and to learn whether their presence and magnitude could be inferred from clinical phenomena. The subject can be approached best through an analysis of the separate categories of disease. Although the patients with Addison's disease were studied under a great variety of conditions, as is evident from the wide variations of [Na], [HCO_3] and [Cl], Δ exceeded 11 meq. in 9 of the 37 observations and was slightly greater than 16 meq. once. In this disorder, therefore, Σ gives a reliable estimate of [Na]. Reductions of either [HCO_3] or [Cl] are referable almost entirely to depletion of [Na]. Only in the most critical states is there any appreciable accumulation in the serum of inorganic phosphate or other anions. The one high figure for Δ, 18.2 meq., in this series was found in a young adult male with implants of pellets of desoxycorticosterone. The observation was made immediately after admission. The patient had suffered from diarrhea and vomiting, he was in a state of collapse, and had been anuric for 24 hours. In addition he was cyanotic and had physical signs and x-ray evidence of bronchopneumonia and atelectasis, confirmed at a subsequent autopsy. [Na], [Cl] and [HCO_3] were, respectively, 141.0, 104.7 and 18.1 meq. per liter. Presumably anuria, circulatory collapse and the pulmonary disease prevented him from compensating more adequately for a starvation ketosis. Although the electrolyte pattern of his serum was rapidly restored to normal by the administration of fluids and measures di-

rected to the support of his circulation, he died 4 days later.

The patients with pulmonary disease fall into 2 categories. In the first are subjects with tuberculosis who resemble patients with Addison's disease in their tendency to waste sodium in the urine (6). In only one of these did Δ exceed 11.0 meq. and then only by 0.2 meq. per liter. In the second category are patients with disorders of the respiratory system, such as asthma, which interfere with the discharge of CO_2. This leads to elevation of $[HCO_3]$ with a compensatory depression of [Cl]. In 2 cases of this type Δ exceeded 16 meq., being 20.1 in both. Both patients were intensely cyanotic; the blood nonprotein nitrogen of one was 49 mgm. per cent. Presumably lactic acid, and possibly other organic acids, were responsible for the high concentrations of organic acid. Such acids would ordinarily displace bicarbonate; but the respiratory disability in these cases prohibited compensation by this reaction. Instead bicarbonate was elevated, in 1 patient on 1 occasion to 56.0 meq. per liter.

The high frequency of excessive values of Δ in diabetic patients is to be expected. The acids are probably in most instances ketone bodies. It may be seen from Figure 1 that there is a fair inverse correlation between Δ and $[HCO_3]$. When the quantity of acid in the circulation increases, 2 reactions are called into play: (*1*) the acids are excreted in the urine; (*2*) bicarbonate is displaced by the acid, while the CO_2 thus released is excreted by the respiratory system. The relative amounts of sodium balanced by bicarbonate and by other acids, then, depend on the efficiency of these 2 processes. If the eliminative mechanisms are operating effectively, there should be some degree of inverse correlation between $[HCO_3]$ and Δ. Such a correlation is evident in the observations from diabetic patients.

In patients with renal disease, high values of Δ coincided regularly with gross nitrogen retention. Usually $[HCO_3]$ was coincidently reduced. These bicarbonate deficits have been attributed to the accumulations in the serum of inorganic phosphate and sulfate, but these were not large enough to

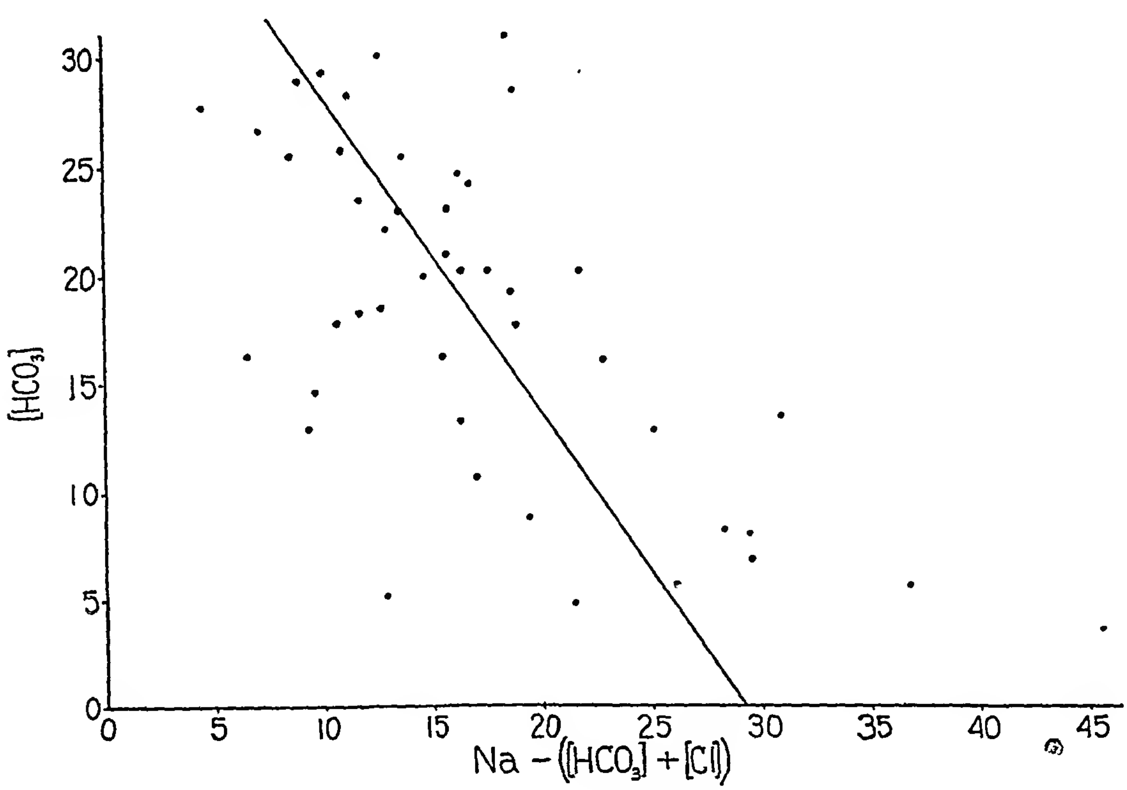

FIG. 1. THE RELATION OF BICARBONATE TO UNDETERMINED ACIDS IN DIABETIC PATIENTS

Values are expressed in milliequivalents per liter. The diagonal line is defined by the equation, $29.31 - 0.685\ [HCO_3] = \Delta$.

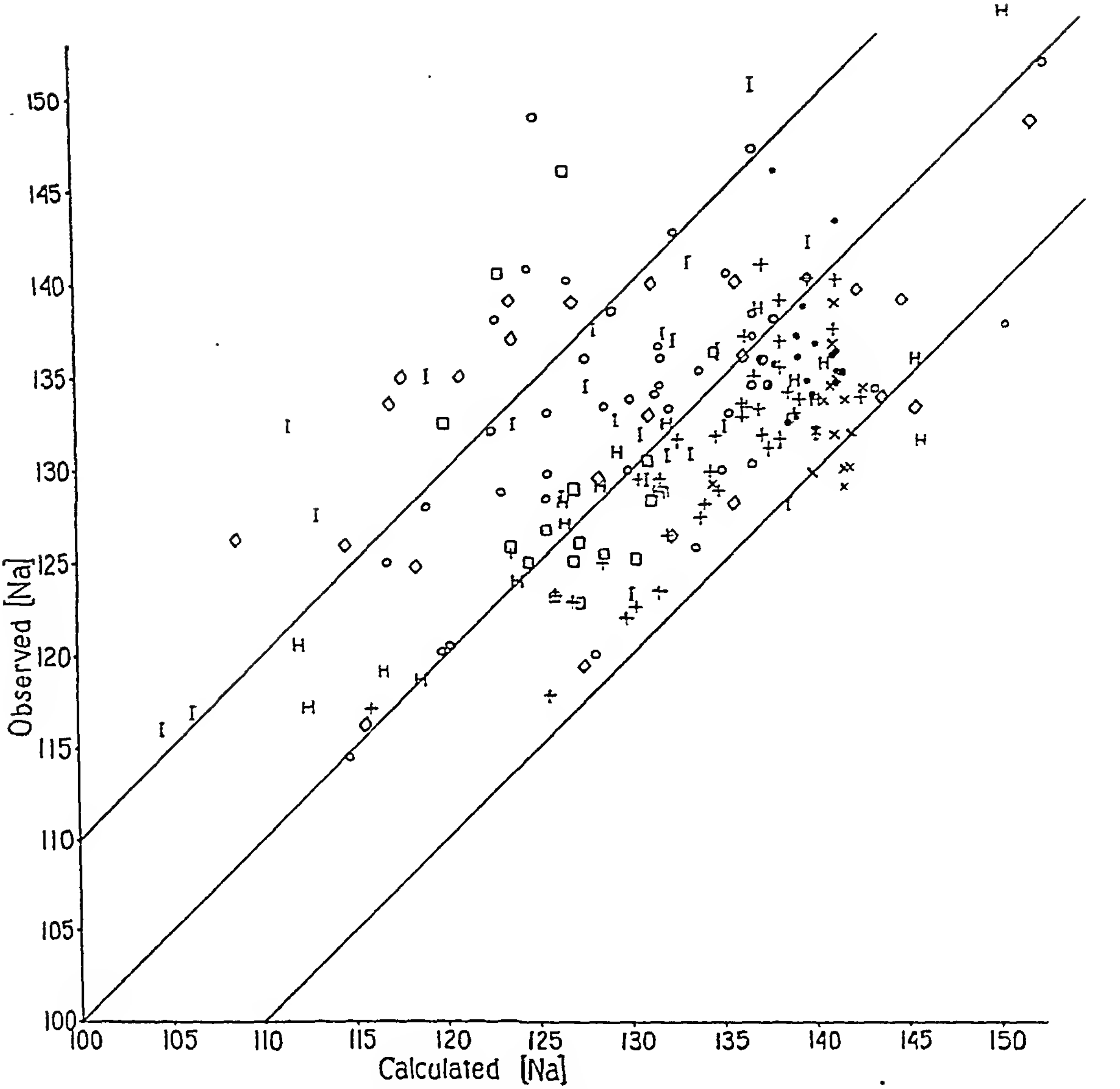

FIG. 2. OBSERVED VALUES OF SERUM [Na] IN MILLIEQUIVALENTS PER LITER COMPARED WITH VALUES CALCULATED BY THE EQUATION,

$$[Cl] + [HCO_3] + 23.2 - 0.50\ [HCO_3] = [Na]$$

The middle diagonal line represents perfect agreement; the 2 lines paralleling it delimit a zone of ± 10 meq. per liter. The point H in the upper right-hand corner should actually be defined by observed [Na] = 167.6, calculated [Na] = 164.4. O = normal male, × = normal female, + = Addison's disease, □ = pulmonary disease, ○ = diabetes mellitus, ◇ = renal disease, H = mercury poisoning, I = miscellaneous disease.

explain both the bicarbonate deficits and the excesses of undetermined acids, which often amounted to 30 meq. per liter or more. Chloride was also reduced in many instances and sodium was seldom elevated. That the high Δ's in renal disease were not referable to impaired renal function alone is suggested by comparison with mercury poisoning. In this condition, in spite of oliguria and high blood nonprotein nitrogen, Δ was usually quite moderate.

The miscellaneous series includes such heterogeneous cases that their analysis as a group cannot be profitable.

The mean line describing the relation of $[HCO_3]$ to Δ in patients with diabetes is defined by the equation, $29.31 - 0.685[HCO_3] = \Delta$, [illegible]

6.61. From Figure 1 it is evident that the distribution of points about this line is somewhat asymmetrical. At high concentrations of $[HCO_3]$ Δ exceeds expectations. When the same line was tested on all observations it did not describe a general relation between abnormal accumulations of anions and $[HCO_3]$. A similar line was, therefore, constructed from all observations. This line is defined by the equation, $23.2 - 0.50[HCO_3] = \Delta$, s.d. $= \pm 7.09$ meq. per liter. With the aid of this relation [Na] can be calculated from $[HCO_3]$ and [Cl] by the equation, $[Cl] + 23.2 + 0.50 [HCO_3] = [Na]$, within $\pm$ 10 meq. per liter in 86 per cent and within $\pm$ 15 meq. in 95 per cent of all observations. The relation between observed and calculated [Na] is shown in Figure 2. Although observed values of Δ are almost always positive, values estimated by the formula are both positive and negative. Some consistent trends are also noticeable. For example, calculated values of [Na] in normal subjects are, with 2 exceptions, greater than observed values. The 2 exceptions are in males. The difference is greater in females than in males. It may be that the line describing the relation of Δ to $[HCO_3]$ is distorted by certain characteristics of one or more pathological groups or that this relationship is actually curvilinear.

Data on the subjects in which the calculated and observed values of [Na] differed from one another by more than 10 meq. per liter are presented in Table II. On 2 occasions in 1 female differences of − 12.4 and − 11.4 meq. were observed, on 3 other occasions the differences in the same subject were − 9.9, −9.9., and − 9.7 meq. per liter (the difference is given the negative sign when the calculated value is greater than the observed value). The observed Δ in the same sera varied from − 1.3 to 3.6. Another of the normal women had a difference of − 11.6 meq. with an observed Δ of 0.4 meq. per liter. These large negative differences were associated with high normal values of [Cl] (106.0 to 106.8 meq. per liter). One other normal female with a [Cl] of 106.8 meq. per liter had a difference of − 8.6 meq. Among the women there is a tendency, not apparent in the men, for the negative error to vary with [Cl], which is consistently greater in females than in males. In the pathological series, calculated [Na] exceeded observed [Na] by more than 10 meq. per liter 4 times, always when [Cl] was greater than 110.0 meq. per liter. In only 1 observation out of 7 in which [Cl] exceeded 110.0 meq. per liter was observed [Na] greater than calculated [Na]. In this case, a diabetic in acidosis, when [Cl] was 122.2 meq. per liter, the difference was 13.3 meq. (observed Δ 1.3 meq.). The observed [Na] on

TABLE II

Observations in which observed [Na] differed from calculated [Na] by more than 10 meq. per liter

Subject	$[HCO_3]$	[Cl]	Σ	[Na] Observed	[Na] Calculated	Δ Observed	Blood NPN	Blood Sugar
	meq. per liter						mgm. per cent	
Normal females								
PMH	24.3	106.0	130.3	129.0	141.4	−1.3		
	24.4	106.0	130.4	130.0	141.4	−0.4		
RC	24.1	106.4	130.5	130.1	141.3	0.4		
Pulmonary disease								
B84061	41.6	78.8	120.4	140.5	122.8	20.1	49	
	56.0	75.1	131.1	146.0	126.3	14.9		
A36064	31.5	80.9	112.4	132.5	119.8	20.1	21	
Diabetes mellitus								
60404	5.7	98.2	103.9	140.7	124.3	36.8	33	588
90339	17.8	118.0	135.8	137.6	150.1	1.8	37	224
42994	3.6	99.8	103.4	148.9	124.8	45.5	42	588
B47553	17.7	101.6	119.3	138.0	122.7	18.7		61
B31839	13.7	96.5	110.2	141.0	126.6	30.8	159	
Renal disease								
A8132	20.7	111.5	132.2	133.2	145.1	1.0	59	
B86920	20.0	81.3	101.3	126.0	114.5	24.7	111	
	10.9	98.1	109.0	139.0	126.8	30.0	168	
	13.3	93.5	106.8	139.0	123.4	32.2	232	
	13.3	90.9	104.2	135.0	120.8	30.8	266	
	12.7	94.0	106.7	137.0	123.6	30.3	278	
	11.7	88.6	100.3	135.0	117.7	34.7	269	
B86008	14.2	86.7	100.9	133.5	117.0	32.6	158	
B90968	13.5	79.7	93.2	126.3	108.7	33.1	166	
Mercury poisoning								
70280	18.5	113.0	131.5	131.5	145.5	0	47	
	16.4	113.8	130.2	135.9	145.2	5.7	54	
Miscellaneous diseases								
A39880	29.0	81.3	110.3	135.0	119.0	24.7	27	
78642	40.9	67.8	108.7	132.4	111.5	23.7	49	
	35.8	71.9	107.7	127.5	113.0	19.8		
B86345	20.8	70.8	91.6	116.0	104.4	24.4	194	
A60444	27.3	99.5	126.8	150.5	136.4	23.7	33	

this occasion was 151.6 meq. per liter. In all other instances high [Cl] was associated with a normal [Na]. It therefore appears that when [Cl] exceeds 110.0 meq. per liter the prediction equation usually overestimates [Na]. If [HCO_3] is depressed by hyperchloremia there is no reason why Δ should be increased. Since the prediction equation is based on the assumption that any depression of [HCO_3] is referable to an increase in the serum of the concentration of anions other than [Cl], it follows that when [HCO_3] is depressed by [Cl] itself the equation will yield too high a value for [Na].

Calculated [Na] was lower than observed [Na] by more than 10 meq. per liter in 2 patients with pulmonary disease. Both had asthma, emphysema, and heart failure. Among the nephritics also, only those with heart failure and pulmonary congestion had large positive differences. This affords an explanation for the absence of such errors in the patients with mercury poisoning, who had no respiratory impairment. Observed [Na] exceeded calculated [Na] by more than 10 meq. in 4 patients with miscellaneous diseases. The first of these, who had encephalitis, at the time of the examination was stuporous, cyanotic, and breathing stertorously. His [HCO_3] was high, [Cl] greatly reduced, with [Na] normal. It may be inferred that he had a primary CO_2 excess. The second case, A78642, besides diabetic acidosis had pyloric obstruction. He was extremely dehydrated and in a state of circulatory collapse. B86345, with a broken neck, had retention of urine and circulatory failure. He was deeply cyanotic, his lungs full of bubbling rales. In all these cases, therefore, a large positive difference was associated with impairment of the respiratory system which interfered with the elimination of CO_2. Invariably [Cl] was reduced; presumably, it was taking over the function of [HCO_3] in making room for the extra load of anions. The latter was also often diminished, especially in the patients with renal disease, but less than it would have been had the respiratory exchange been unimpaired. Consequently the usual inverse proportionality between [HCO_3] and Δ was not maintained. Respiratory insufficiency in these cases was not absolute, but relative.

The last patient, A60444, was in an active stage of familial periodic palsy with a serum potassium of 1.7 meq. per liter. Whether this interfered with his respiratory exchange, it is impossible to say because the analysis was made for routine diagnostic purposes and associated disturbances of his vital signs were not noted.

Of the diabetic cases, 90339 with high [Cl] has already been mentioned among those who had negative differences with hyperchloremia. In the remaining 4 observed [Na] exceeded calculated [Na] by 14 to 24 meq. per liter. Two of these patients, 60404 and 42994, had extreme acidosis and dehydration. It may be that the respiratory system had to assume more than its usual share of the burden because the kidneys were unable under these circumstances to work efficiently. On the other hand, it may be, as was suggested above, that the relation of [HCO_3] to Δ is actually curvilinear. At normal [HCO_3] the prediction equation seemed to exaggerate [Na]; at extremely low [HCO_3] it may yield values that are too low. Errors of this nature and magnitude in extreme diabetic acidosis would not, however, lead to improper impressions or therapy. The other 2 patients had frank evidences of heart failure; in B31839 this was complicated by bronchiectasis, asthma, and emphysema.

It appears, therefore, to be possible to estimate serum [Na] within $\pm$10 meq. per liter from [HCO_3] + [Cl] with the aid of clinical observation. In such estimations account must be taken of the fact that as anions other than [HCO_3] and [Cl] accumulate in the serum Δ increases in inverse proportion to the depression of [HCO_3]. A prediction formula has been devised by which these increases of Δ may be calculated from [HCO_3]. In at least 2 conditions this formula fails. If [HCO_3] is displaced by [Cl] it yields excessively high calculated values for [Na]; if the respiratory elimination of CO_2 is impaired it yields erroneously low values.

There can be no doubt that direct measurement of [Na] will give more precise information; but no other combination of any 2 of the 3 variables, [Na], [HCO_3] and [Cl], will be as serviceable as [HCO_3] + [Cl]. The combination of [Na] and [HCO_3] would obviously be most inadequate because the difference between these 2 would include the largest anion fraction, [Cl], as well as Δ. In diabetic acidosis and other conditions, such as exercise or anoxemia, in which Δ is composed of combustible organic anions, measurements of [Na] and [Cl] would give useful information. In these

conditions it could be assumed that the whole difference, [Na] − [Cl], was potentially bicarbonate, since the sodium liberated by the combustion of these foreign acids would form bicarbonate with CO_2. In renal insufficiency, however, when phosphate and sulfate accumulate in the serum, [Na] − [Cl] would have an altogether different significance and would give imperfect information.

SUMMARY

A prediction formula has been devised by which the concentration of sodium in blood serum may be estimated within ± 10 meq. per liter from the concentrations of CO_2 and chloride. Two conditions have been found in which this formula is inapplicable. In hyperchloremia it yields erroneously high values; when the respiratory elimination of CO_2 is impaired it yields erroneously low values. Since these sources of error are readily detectable they do not detract greatly from the value of the formula.

BIBLIOGRAPHY

1. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Methods. Williams and Wilkins Co., Baltimore, 1931.
2. Hald, P. M., Notes on the determination and distribution of sodium and potassium in cells and serum of normal human blood. J. Biol. Chem., 1946, 163, 429.
3. Hald, P. M., The flame photometer for the measurement of sodium and potassium in biological materials. J. Biol. Chem., 1947, 167, 499.
4. Hastings, A. B., Sendroy, J., Jr., and Van Slyke, D. D., Studies of gas and electrolyte equilibria in blood. XII. The value of pK in the Henderson-Hasselbalch equation for blood serum. J. Biol. Chem., 1928, 79, 183.
5. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Interpretations. Williams and Wilkins Co., Baltimore, 1931.
6. Winkler, A. W., and Crankshaw, O. F., Chloride depletion in conditions other than Addison's disease. J. Clin. Invest., 1938, 17, 1.

THE TRANSPORT AND EXCRETION OF URIC ACID IN MAN. I. TRUE URIC ACID IN NORMAL CEREBROSPINAL FLUID, IN PLASMA, AND IN ULTRAFILTRATES OF PLASMA

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Human cerebrospinal fluid has been reported to contain a relatively small concentration of uric acid compared to that of plasma (*cf.* Table II). However, it is not known what proportion of these reported uric acid values is due to chromogenic substances similar to those giving the color reaction for uric acid in plasma (1). The introduction of enzymatic methods for determining uric acid specifically (2 to 5) has made reexamination of spinal fluid values advisable, particularly with regard to their relation to plasma ultrafiltrates.

SUBJECTS

Cerebrospinal fluids[2] were obtained by lumbar puncture from individuals suspected of neurosyphilis and from others undergoing routine follow-up after antiluetic therapy. Those fluids which were serologically and Pandy negative were combined into 10-ml. pools, each pool representing 5 individuals. Total protein was then determined on each pool and those with a content of less than 35 mgm. per 100 ml. were considered satisfactory for study.

Ventricular fluids were obtained during diagnostic ventriculography on neurological patients without mass lesions or inflammatory intracranial disease.

Plasma for ultrafiltration was obtained from 2 normal subjects on diets of varying purine content. The results quoted in the text are the average of 8 determinations on one subject and 5 on the second.

METHODS

Uric acid was determined in plasma, plasma ultrafiltrates, and in cerebrospinal fluid by a modification of the method of Buchanan, Block and Christman (2) for the Evelyn photoelectric colorimeter.

The essentials of this determination and the terms used in reporting results are as follows:

1. A 1-ml. aliquot of the specimen is deproteinated and the color developed with the arsenophosphotungstic acid and urea-cyanide reagents. The concentration of uric acid equivalent to the color developed is termed *total urate*.
2. Another (2-ml.) aliquot of the specimen is treated with uricase at pH 9.1 to destroy the uric acid present. It is then treated as above, the color developed being termed *chromogen*.
3. The difference between the values for total urate and for chromogen represents the amount of uric acid destroyed by the specific enzyme and is therefore termed *true urate*.

The actual size of the aliquots given in the preceding paragraph is that used in plasma determinations; cerebrospinal fluid contains so little urate that it is advisable to use twice or 3 times these amounts.

The nature of the chromogenic substances in plasma and urine which give the color reaction for urate has been discussed recently (1) and will be reviewed more comprehensively in a forthcoming publication (6). A portion of the chromogen is non-purine in origin, but another portion is due to an incompletely characterized purine metabolite (or metabolites) of endogenous origin. In addition, methylurates from the methylxanthines of dietary origin contribute to the chromogen.

The normal plasma urate values quoted are based upon repeated determinations on 31 normal individuals and are similar to the values obtained by Bulger and Johns (3) with a uricase method.

Plasma for ultrafiltration was obtained from blood drawn in a heparinized syringe and immediately centrifuged; no attempt was made to preserve the normal carbon dioxide content. Ultrafiltrates of plasma were obtained by a modification of the method of Lavietes (7). The membranes used were DuPont No. 600 cellophane, soaked 3 days at 0° before used.[3]

RESULTS

The results of the urate partition in pooled normal cerebrospinal fluid are given in Table I, together with the average normal concentrations in plasma and the calculated C.S.F./plasma ratios. These data show that normal human cerebrospinal fluid appears to contain, on the average, only 6

[1] The department is in part supported by the Michael Reese Research Foundation.

[2] The authors are indebted to the Hektoen Institute for Medical Research of Cook County Hospital for cooperation in obtaining cerebrospinal fluid specimens.

[3] The cellophane membranes used in ultrafiltration were made available through the cooperation of E. I. du Pont de Nemours Co.

TABLE I

Urate content of normal human cerebrospinal fluid and calculated average C.S.F./plasma ratio

	Total urate	Chromogen	True urate	Total protein
	mgm. per 100 ml.	*mgm. per 100 ml.*	*mgm. per 100 ml.*	*mgm. per 100 ml.*
C.S.F. pools				
1	0.58	0.26	0.32	20
2	0.35	0.28	0.07	25
3	0.80	0.60	0.20	30
4	0.58	0.38	0.20	13
5	0.76	0.33	0.43	10
6	0.62	0.38	0.24	13
7	0.51	0.38	0.13	13
8	0.67	0.38	0.29	14
9	0.59	0.33	0.26	13
Average C.S.F. concentration	0.61	0.37	0.24	
Average plasma concentration	4.76	0.74	4.02	
Average C.S.F./plasma ratio	0.13	0.50	0.06	

TABLE II

Plasma and cerebrospinal fluid total urate values in various investigations

Authors	Source of material	Average C.S.F.	Average serum or blood	C.S.F./Blood	[illegible]
		mgm. per 100 ml.	*mgm. per 100 ml.*		
Present investigation	Pooled normal specimens	0.61	4.76 plasma		0.1[illegible]
Cantarow and Trumper (8)	Normal individuals	0.63	3.00 blood	0.210	0.15 Estima[illegible]
Reiche (9)	230 medical patients	1.23	4.16 blood	0.237	0.215 Estimat[illegible]
Frada (10)	9 medical patients	0.89	4.82 blood	0.185	0.137* Estimated
Ingvarsson (11)	33 schizophrenic patients	0.47	2.79 blood	0.168	0.126* Estimated
Katzenellenbogen (12)	20 schizophrenic patients	0.19	2.49 blood	0.076	0.057* Estimated
Cockrill (13)	5 normal individuals	1.83	4.53 plasma		0.404
Chauffard, Brodin and Grigaut (14)	4 medical patients	0.54	4.81 serum		0.112
Talbott (15)	12 gouty patients	1.79	7.26 serum		0.246

* Depending chiefly upon the hematocrit and upon the type of whole blood method used, C.S.F./blood ratios will average 135 to 200 per cent of C.S.F./plasma ratios. To facilitate comparison of data, where authors determined urate in blood the C.S.F./plasma ratio in the final column has been estimated as 0.75 (C.S.F./blood).

per cent of the true urate present in plasma. Furthermore, application of the uricase method to spinal fluid shows that slightly less than half the chromogenic material present is true urate.

Our results for total urate in cerebrospinal fluid are comparable to those found in previous investigations, which are summarized in Table II. The values found by Katzenellenbogen (12) are somewhat lower than those in other studies because he employed a silver precipitation method which yields values approximately equivalent to true urate under suitable circumstances (1). Normal values in children are reported to be 50 per cent higher than in adults (16).

The C.S.F./plasma ratios reported by different investigators show considerable variation; but most recent studies show a mean ratio of 0.1 to 0.2 for total urate. Reiche (9), by dividing his large material into 3 groups, was able to show a rough parallelism between the blood urate level and the cerebrospinal fluid level.

We have secured a small number of ventricular fluids from patients without mass lesions of the brain or inflammatory intracranial disease. These data, presented in Table III, show merely that the urate content and partition in ventricular fluid do not differ strikingly from that obtained by lumbar puncture.

The difference between plasma and cerebrospinal fluid urate concentrations is so large that it

TABLE III

Urate concentration and partition in ventricular fluid obtained from patients without mass lesions or inflammatory intracranial disease

Sample number	Diagnosis	Total urate	Chromogen	True urate	Protein
		mgm. per 100 ml.	*mgm. per 100 ml.*	*mgm. per 100 ml.*	*mgm. per 100 ml.*
V2	Cerebral atrophy, etiology unknown	1.30	0.90	0.40	19
V3	Chronic internal hydrocephalus	0.50	0.10	0.40	20
V4	Undiagnosed lesion, region of optic chiasm	0.70	0.50	0.20	34
V5	Arteriosclerotic cerebral vascular disease	0.70	0.30	0.40	19
	Average	0.80	0.45	0.35	23

TABLE IV

Ultrafiltrability of urate fractions through cellophane in 2 normal subjects (10)

	Subjects	
	W. Q. W.*	H. B. W.†
Plasma total urate, *mgm. per 100 ml.*	7.13	4.52
Ultrafiltrate total urate, *mgm. per 100 ml.*	5.29	3.78
Per cent ultrafiltrable	74.3	83.6
Plasma chromogen, *mgm. per 100 ml.*	1.41	0.92
Ultrafiltrate chromogen, *mgm. per 100 ml.*	1.12	0.85
Per cent ultrafiltrable	79.2	92.4
Plasma true urate, *mgm. per 100 ml.*	5.72	3.60
Ultrafiltrate true urate, *mgm. per 100 ml.*	4.17	2.93
Per cent ultrafiltrable	72.9	81.4

* Average of 8 experiments, 3 of which were during high purine diet periods.
† Average of 5 experiments, all on normal diet.

clearly cannot be explained on the basis of the Donnan phenomenon. Since the only other clearly established cause for such a discrepancy between spinal fluid and plasma constituents is nondiffusibility, ordinarily due to the binding of a substance to plasma protein, we attempted to determine the degree of protein binding of urate by pressure ultrafiltration through cellophane.

Adlersberg, Grishman and Sobotka (17) have reviewed the literature dealing with attempts to demonstrate protein binding of urate. In their own experiments, they found 84 per cent of the plasma total urate ultrafiltrable in normal individuals.

Our experience with ultrafiltration confirms that of Adlersberg and his coworkers. We found 79 per cent of the plasma total urate and 77 per cent of the plasma true urate to be free to ultrafiltration (Table IV). It is obvious that the quantities of true urate in normal cerebrospinal fluid are much less than those which appear to be free in plasma when cellophane ultrafiltration is used to indicate the degree of plasma binding.

DISCUSSION

The data presented above demonstrate the cerebrospinal fluid true urate concentration to be only 6 per cent of that present in plasma. The question therefore arises by what mechanism such an unequal distribution may be maintained.

Cerebrospinal fluid, it is generally agreed, resembles a modified ultrafiltrate of plasma (8, 18, 19). It appears to be subject to alterations predicted by the Gibbs-Donnan rule. Various substances are present in relatively small amounts because of plasma binding and, to a lesser extent, because they may circulate in plasma water in a colloidal state (calcium, bilirubin, cholesterol, salicylate). The quantity of magnesium in cerebrospinal fluid is greater than in plasma and the difference is magnified when plasma binding is considered; it has, however, been suggested that magnesium is a brain metabolite. There remain differences in potassium, glucose, and phosphate partition which cannot be well explained at present.

When true uric acid is considered, there appears to be little resemblance between cerebrospinal fluid and plasma ultrafiltrates. The ultrafiltrate contains 77 per cent of the uric acid concentration of plasma, while the cerebrospinal fluid contains only 1/10 as much urate as the ultrafiltrate.

Three possibilities have been suggested to explain the peculiarity in urate distribution; they will be briefly listed without extended discussion. The first is that true uric acid enters the cerebrospinal fluid in somewhat greater concentration and is converted to substances not fermentable by uricase. Since a small series of determinations of urate partition in ventricular fluid show no differences from fluid obtained by lumbar puncture, this appears not to be tenable.

The second possibility is that the modified ultrafiltration theory of cerebrospinal fluid formation does not apply to true uric acid. Reiche's (9) demonstration of parallelism between blood and spinal fluid urate concentrations makes this appear improbable.

The third possibility is that ultrafiltration *through cellophane* does not truly reflect the diffusibility of urate through biological membranes. Such behavior is observed in the case of p-aminohippuric acid which shows much greater binding in clearance studies than in ultrafiltration studies (20). From electrophoretic experiments, Bennhold (21) concluded that uric acid is bound to plasma albumin by a linkage highly labile to pressure and that the degree of plasma binding was

much greater than could be shown by ultrafiltration. Although it is not clear that non-diffusibility is entirely due to binding to plasma protein, evidence obtained from a study of renal and other biological functions (6, 22) suggests that the true urate content of the cerebrospinal fluid may be essentially equal to the plasma-free true urate.

SUMMARY

The concentration of true uric acid in normal human cerebrospinal fluid has been determined enzymatically. Substances giving the arsenophosphotungstate color reaction for urate are present in considerable amount, but true urate forms only 40 per cent of these substances. The concentration of true urate in normal spinal fluids averages 6 per cent of that in normal plasma.

Approximately 77 per cent of the plasma true urate is ultrafiltrable through a cellophane membrane under pressure, but spinal fluid contains only $\frac{1}{10}$ the true urate which is found in a plasma ultrafiltrate.

The various possible explanations of these findings are briefly considered.

BIBLIOGRAPHY

1. Levine, R., Wolfson, W. Q., and Huddlestun, B., A purine metabolite in plasma, which gives the same color reaction as uric acid. Proc. Am. Federation Clin. Research, 1945, 2, 56.
2. Buchanan, O., Block, W. D., and Christman, A. A., Metabolism of the methylated purines. I. Enzymatic determination of urinary uric acid. J. Biol. Chem., 1945, 157, 161.
3. Bulger, H. A., and Johns, H. E., The determination of plasma uric acid. J. Biol. Chem., 1941, 140, 427.
4. Blauch, M. B., and Koch, F. C., A new method for the determination of uric acid in blood, with uricase. J. Biol. Chem., 1939, 130, 443.
5. Schaffer, N. K., The determination of uric acid in urine with crude uricase. J. Biol. Chem., 1944, 153, 163.
6. In preparation.
7. Lavietics, P. H., Anaerobic ultrafiltration. J. Biol. Chem., 1937, 120, 267.
8. Cantarow, A. and Trumper, M., Clinical Biochemistry, W. B. Saunders Co., Philadelphia, 1945, Third Edition, pp. 102, 508–513.
9. Reiche, F., Die Harnsäure im Blut und Liquor und in pathologischen Flüssigkeiten. Ztschr. f. klin. Med., 1933, 125, 565.
10. Frada, G., Sul contenuto in acido urico del liquido cerebro-spinale. Biochem. e terap. sper., 1938, 25, 464.
11. Ingvarrson, G., Eine Studie über den Harnsäuregehalt des Liquor cerebrospinalis bei einigen psychotischen Zuständen. Acta psychiat et neurol., 1937, 12, 61.
12. Katzenellenbogen, S., Studies in schizophrenia; chemical analyses of blood and cerebrospinal fluid. Arch. Neurol. & Psychiat., 1937, 37, 881.
13. Cockrill, J. R., Nonelectrolytes; their distribution between the blood and the cerebrospinal fluid. Arch. Neurol. & Psychiat., 1931, 25, 1297.
14. Chauffard, A., Brodin, P., and Grigaut, A., Diffusibilité clinique comparée de l'acide urique et de l'urée. Compt. Rend. Soc. Biol., 1922, 86, 355.
15. Talbott, J. H., Gout. Oxford University Press, New York, 1943.
16. Cantarow, A. A., Personal communication.
17. Adlersberg, D., Grishman, E., and Sobotka, H., Uric acid partition in gout and in hepatic disease. Arch. Int. Med., 1942, 70, 101.
18. Flexner, L. B., The chemistry and nature of the cerebrospinal fluid. Physiol. Rev., 1934, 14, 161.
19. Katzenellenbogen, S., The Cerebrospinal Fluid and its Relation to the Blood. Johns Hopkins Press, Baltimore, 1935.
20. Smith, H. W., Finklestein, N., Aliminosa. L., Crawford, B., and Graber, M., The renal clearances of substituted hippuric acid derivatives and other aromatic acids in the dog and man. J. Clin. Invest., 1945, 24, 388.
21. Bennhold, H., Kylin, E., and Rusznyak, S., Die Eiweisskörper des Blutplasmas. Theodor Steinkopf, Dresden, 1938.
22. Levine, R., and Wolfson, W. Q., What is gout? Newer knowledge of pathological physiology. Proc. Inst. Med. Chicago, 1946, 16, 235.

THE TRANSPORT AND EXCRETION OF URIC ACID IN MAN. II. THE ENDOGENOUS URIC ACID–LIKE CHROMOGEN OF BIOLOGICAL FLUIDS

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Within recent years, enzymatic methods for the determination of true urate have become available. The introduction of such methods by Blauch and Koch (1, 2) has made it possible to estimate true urate in biological material with a high degree of accuracy (3 to 10).

All uricase methods involve the colorimetric determination of uric acid on two aliquots of a sample, one of which has been treated with uricase to destroy the true uric acid present. The apparent concentration of uric acid in the untreated sample, as determined colorimetrically, is referred to as "total urate"; and the concentration remaining after treatment with uricase is referred to as the "urate-like chromogen" (or briefly, "chromogen"). By substracting the concentration of chromogen from that of total urate, the concentration of "true urate" destroyed by the enzyme is obtained.

Most authors have tacitly assumed that the chromogen was merely an interference with the maximal accuracy of urate determination, but the results we obtained appear to require another explanation. A preliminary report on some of this data has appeared elsewhere (11) and this paper is chiefly concerned with the results of our investigations dealing with the chemical nature and physiological significance of the urate-like chromogen.

METHODS

Total urate, true urate, and chromogen are estimated by our modification of the method of Buchanan, Block, and Christman (3) for the Evelyn photoelectric colorimeter. Details of other chemical and physiological methods and complete data on our normal human subjects and gout patients summarized below will appear in a later publication (12).

Urate solutions for continuous intravenous injection in normal human subjects were prepared by dissolving uric acid in 0.05 N sodium hydroxide. The solutions were neutralized and sterilized by Berkfeld filtration. Only a few such experiments were performed as we, like a number of previous workers, have found these solutions to produce frequently unpleasant reactions, chiefly nausea and vomiting. These reactions could not be ascribed to any possible decomposition products since 100 per cent of the added urate was recoverable as true urate by analysis, even after standing for 1 to 2 days. Nucleic acid was given orally in 1.0-gram gelatin capsules; doses as high as 20 grams appear to be fairly well tolerated. Below 10 grams, there are no untoward effects; but single doses in the 10- to 20-gram range may cause transient malaise, hypotension, and vasodilation in the second hour, presumably attributable to adenosine. Other solutions of purines used in animal experiments were made up in .05 N NaOH.

All determinations were done on plasma or serum rather than on whole blood. Although the Folin uric acid precipitation is supposed to be an "unlaked blood" method (*i.e.* the red cells are precipitated without hemolysis) this is not true (13). In addition, the red cells contain a large amount of ergothionine which gives considerable urate color.

TABLE I

Effect of fasting on plasma total urate, chromogen and true urate concentrations in normal human subjects and in dogs

	Total urate	Chromogen	True urate
	mgm. per 100 ml.	*mgm. per 100 ml.*	*mgm. per 100 ml.*
Human subjects			
HBW, 1 hour post-prandial	4.0	0.6	3.4
HBW, 24 hours fasting	4.7	0.6	4.1
WQW, 1 hour post-prandial	6.4	0.7	5.7
WQW, 24 hours fasting	6.6	0.7	5.9
Dogs			
Dog 2, postabsorptive	1.1	0.4	0.7
Dog 2, 3 days fasting	1.1	0.7	0.4
Dog 230, postabsorptive	0.9	0.5	0.4
Dog 230, 3 days fasting	1.1	0.5	0.6

[1] The department is in part supported by the Michael Reese Research Foundation.

RESULTS

The endogenous origin of chromogen. Table I shows the effect of fasting upon the plasma concentrations of chromogen in man and in the dog. These results, which show that chromogen concentrations do not decline upon fasting, indicate the endogenous origin of this substance or group of substances.

The known urate chromogens. A large number of substances (Table II) are not attacked by uricase but do give color in the uric acid reaction. It is, however, possible to conclude that most of these substances cannot be responsible for the observed plasma concentrations of chromogen.

The non-purine substances with large chromogenic equivalents (ascorbate, resorcinol, cystine, glutathione, and ergothionine) are not present either in plasma or urine in concentrations large enough to account for the amounts of chromogen present. (See Table II.) In addition, the data of Buchanan, Block and Christman (3, 4) appear to indicate that the endogenous chromogen is not precipitated by their silver reagent which precipitates ergothionine quantitatively.

TABLE II*

Ability of certain substances to produce color with the uric acid reagents, with known chromogenic equivalents†

Substances which give color	Chromogenic equivalent
1-methyluric acid‡	1.05
Uric acid‡	1.00
1,3-dimethyluric acid	0.51
Ascorbic acid§	0.44
Resorcinol	0.32
3-methyluric acid	0.31
Ergothionine‡	0.24
Uric acid-9-riboside	0.17
1,7-dimethyluric acid	0.02
Cystine	0.005
Glutathione	0.005
3,7-dimethyluric acid	0.004
1,3,7-trimethyluric acid	trace
7-methyluric acid	trace
6-amino-2,8-dihydroxy-purine	unknown

Substances which do not give color	
Adenine	6-amino-8-hydroxy purine
Guanine	8-hydroxy-purine
Xanthine	6-amino-2-hydroxy purine
Hypoxanthine	2-thioxanthine
Methylxanthines	Allantoin
Oxyacetylene-diurein-carboxylic acid	

* Data chiefly from Buchanan, Block and Christman (3, 4), Booth (29), and Falconer and Gulland (30). We have confirmed the chromogenic equivalents for the methyluric acids and for resorcinol reported by Buchanan, Block and Christman (3, 4). From the observation that uricase action upon uric acid gives rise only to non-chromogenic products, it is inferred that oxyacetylene-diurein-carboxylic acid, the chief reaction product (*31*), is non-chromogenic.

† The *chromogenic equivalent* is the number of milligrams of uric acid required to give a color equal in intensity to that produced by 1.0 milligram of the substance being tested.

‡ Precipitated by silver reagents.

§ When ascorbic acid is added to plasma samples treated routinely with no special precautions to prevent oxidation, the chromogenic equivalent is approximately 0.20.

On the other hand, the methylxanthines of dietary beverages (theophylline, theobromine, caffeine), when oxidized in intermediary metabolism, give rise to methylurates of considerable chromogenic value (4, 14). Coffee, tea, and chocolate, however, can hardly account for the plasma and urine chromogens in the animal, in the fasting human, or in subjects on a purine-free diet.

One substance may deserve more study than it has yet received. Some years ago, Minkowski and other workers (15, 16) found that when large amounts of adenine were fed to animals, a purine derivative was deposited in the cells of the renal tubule. Raska (17) has recently made a similar observation. Upon analysis, this purine proved to be 6-amino-2,8-dihydroxy-purine. It is known to give color with the uric acid reagents and its precursor, oxyadenine (6-amino-2-oxy-purine), has been found in pig blood (18). We are aware of no studies indicating either its occurrence or absence in human intermediary metabolism.

It is apparent that if the endogenous chromogen is of purine origin, as we shall attempt to show, it must be very similar to uric acid in order to give appreciable color. The minimum criteria appear to be the presence of 2 oxygen atoms in the 2, 6, and 8 positions with another radical in the third position or 3 oxygen atoms in these loci. Most of the substituted uric acid derivatives, however, give considerably less color than urate itself. The breakdown products of uric acid are not chromogenic, and precursors as similar as the dihydroxy-purines also are not chromogenic.

Since it is possible on these most elementary chemical considerations tentatively to eliminate most known urate derivatives, the endogenous chromogen appears to be a substance the composition of which is as yet not known.

Occurrence and distribution of chromogen. In Table III we have summarized a large body of data concerning the values for true urate and chromogen in various biological fluids (19). It is ap-

TABLE III

*Urate partition in certain biological fluids**

	Number of subjects	Total urate	Chromogen	True urate	True urate / Total urate: Average	True urate / Total urate: S.D.
Human subjects						
Plasma, normal	31†	4.76	0.74	4.02	0.846	±0.032
Plasma, gout	17†	8.82	0.88	7.94	0.900	±0.025
Urine, normal	13	.52	.08	.44	0.850	±0.097
Urine, gout	12	.40	.07	.33	0.840	±0.056
Clearance, normal	13	10.53	8.55	11.00	1.044	±0.137
Clearance, gout	12	5.79	6.07	5.74	0.992	±0.097
C.S.F., normal	9‡	0.67	0.41	0.26	0.392	±0.137
Dogs						
Plasma, normal	9	1.03	0.60	0.43	0.419	±0.164

* Plasma and C.S.F. values are milligrams per 100 ml. Urine values are milligrams per minute per 1.73 sq. m. Clearance values are ml. of plasma cleared per minute per 1.73 sq. m.

† Average plasma values were obtained on larger groups of subjects, both normal and gouty, than were urine and clearance values. Urine and clearance results quoted were obtained simultaneously and in the same individuals.

‡ The data for normal C.S.F. are those for pooled normal fluids (10).

parent that the chief constituent of the total urate values in human plasma and urine, both in normals and in persons with gout, is true urate. In fact, the proportion of total urate formed by true urate is so regular that it may safely be estimated by a conversion factor. Similarly, there is little difference between the total urate and true urate clearance, a point of importance when considering the results of earlier investigation.

The clearance of chromogen is similar in magnitude to that of true urate, and is reduced in gout as is that of true urate, observations which again point to its resemblance to uric acid and certainly suggest it to be a purine derivative.

However, in normal human cerebrospinal fluid (10) and in dog plasma (19), the situation is somewhat different. Here, chromogen forms over 50 per cent of total urate values, and the ratio of true urate to total urate is sufficiently irregular so that it appears hazardous to employ a conversion factor.

Observations on ultrafiltrates of human plasma ([10], Table III) show considerably less plasma binding of chromogen than of true urate, when cellophane ultrafiltration is used as the criterion of binding. Moreover, the C.S.F./plasma ratio for chromogen is considerably greater than for true urate ([10], Table I).

We have been fortunate enough to observe a patient with chronic monoblastic leukemia and gout who had an output of urate of 2.95 mgm. per minute or about 6 times normal. In this patient, both true urate and chromogen were excreted in increased amounts, the chromogen excretion being about 4 times normal.

Site of origin of chromogen. In the dog, rabbit, and monkey, removal of the liver (or evisceration), is followed by a rise in plasma uric acid owing to the removal of hepatic uricase which normally converts uric acid to allantoin (20 to 24). If the renal pedicles are ligated, or the kidneys removed, excretion of urate and allantoin is prevented, making it possible to study the peripheral production of urate by observation of the plasma uric acid level. In this eviscerated-bilaterally-nephrectomized preparation, the rise in plasma urate, at least for the first few hours postoperatively, is linear, indicating that, either reduction of urate to its precursors is not affected by rising plasma levels or that breakdown rates and resynthesis rates are so balanced as to mask this effect.

In the eviscerated-bilaterally-nephrectomized dog (Table IV), operation is followed by a rise in both true urate and chromogen. This appears actually to depend upon the rate at which urate precursors are made available, since the injection

TABLE IV

Effect of evisceration and bilateral nephrectomy upon plasma urate partition in the dog

The data are average results of 5 experiments.

	Total urate	Chromogen	True urate	True urate / Total urate
	mgm. per 100 ml.	*mgm. per 100 ml.*	*mgm. per 100 ml.*	
Preoperative	0.99	0.60	[illegible]	[illegible]
Postoperative				
0.0 hour	1.53	0.70	[illegible]	[illegible]
1.5 hours	3.00	1.03	[illegible]	[illegible]
3.0 hours	4.33	[illegible]	[illegible]	[illegible]

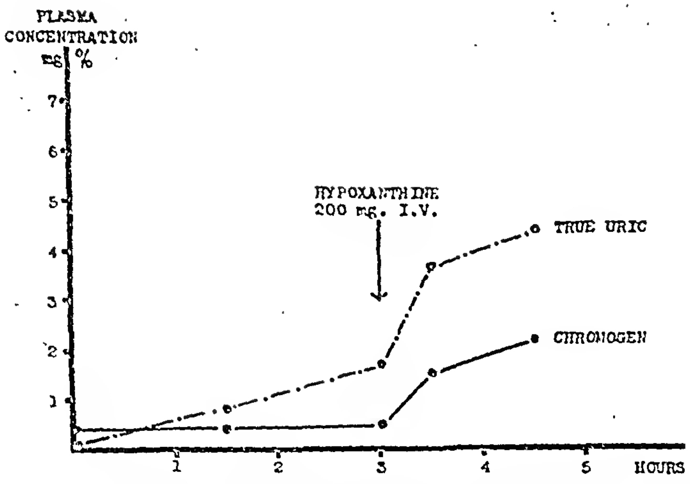

FIG. 1. EFFECT OF HYPOXANTHINE INJECTED INTRAVENOUSLY IN HEPATECTOMIZED DOG

of xanthine or hypoxanthine (Figure 1) into this preparation is followed by a rapid, simultaneous rise in both plasma true urate and plasma chromogen. However, while these data indicate that chromogen may be formed in the peripheral tissue, they do not exclude the possibility that it is derived from urate.

Effect of urate and its precursors in man. In man, the oral administration of large doses of nucleic acid (Figure 3) leads to increased excretion of both true urate and chromogen. Freyberg, Block and Geib (25) have shown that administration of adenine or guanine has similar effects.

Man, however, is particularly suitable for the study of the possibility that chromogen may be formed from urate in the peripheral tissue since he lacks the hepatic uricase of the dog. The results of this experiment (Figure 2) showed that the continuous intravenous injection of urate solutions leads to a rapid rise in both plasma true urate and chromogen. Since it is possible to recover 100 per cent of urate added to plasma or blood *in vitro,* one must conclude that some system, presumably enzymatic, located in the peripheral tissue, or located in the blood and very susceptible to spontaneous breakdown, is responsible.

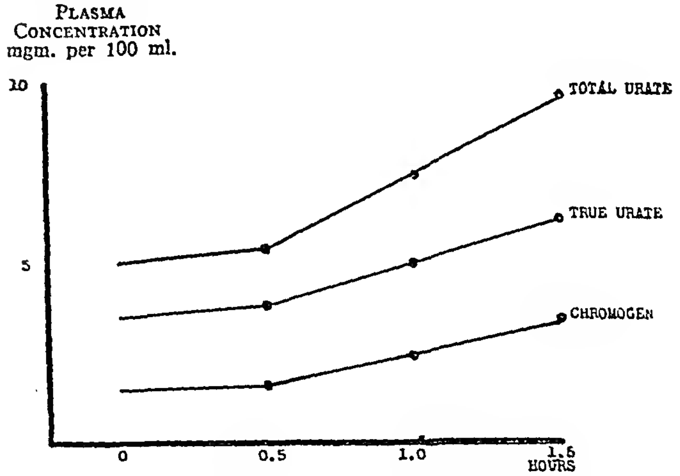

FIG. 2. EFFECT OF CONTINUOUS INTRAVENOUS INFUSION OF SODIUM URATE IN HUMAN

One-half gram of sodium urate solution, 100 mgm. per 100 ml., was injected between 0.5 and 1.5 hours.

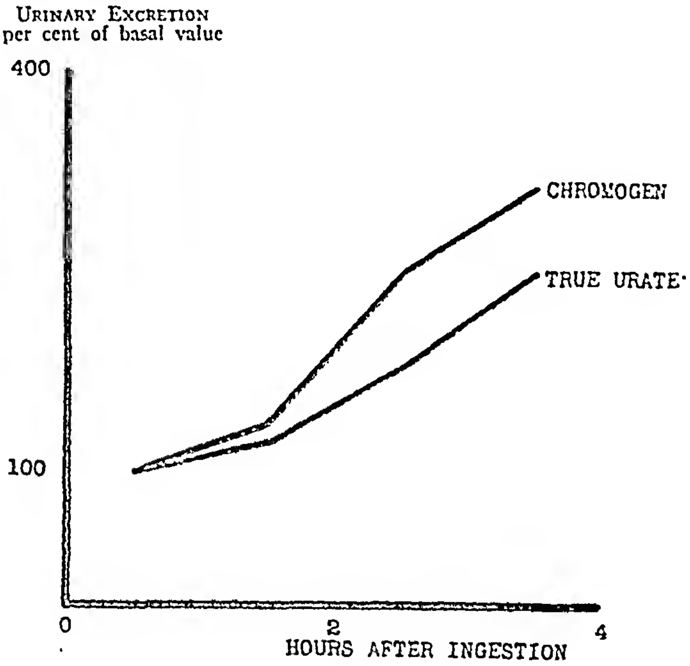

FIG. 3. EFFECT OF A SINGLE 15.0-GRAM ORAL DOSE OF NUCLEIC ACID UPON THE URINARY EXCRETION OF TRUE URATE AND OF CHROMOGEN

Effect of uricosuric drugs on chromogen excretion. Another similarity between urate and chromogen lies in the response to the uricosuric group of drugs, those agents which increase urate output in urine and decrease its plasma level. All the uricosuric drugs so far investigated (cincophen,

TABLE V

Effect of diodrast upon urine urate partition in a normal human subject

Ten ml. of 35 per cent diodrast were injected intravenously at the end of the first hour, and at the end of the second hour.

Hour	Total urate	Chromogen	True urate
	mgm. per min.	*mgm. per min.*	*mgm. per min.*
1	0.64	0.13	0.51
2	2.66	0.31	2.35
3	2.09	0.20	1.89
4	1.07	0.18	0.89

salicylate, diodrast) have been found to increase the output of chromogen as well as that of true urate (26). The results of a typical experiment with diodrast are shown in Table V.

The chromogen content of a gouty tophus. Because of the many similarities in the physiological behavior of true urate and chromogen, we were interested to see whether chromogen, like true urate, is deposited in the gouty tophus.

A large surgically removed tophus was made available for analysis.[2] This analysis (Table VI) was distinctly surprising in that it showed the virtual absence of chromogen.

TABLE VI

Analysis of surgically removed tophus

	grams
Weight of sample	5.500
Water	3.100
Connective tissue	1.000
Total urate	0.681
Chromogen	Negligible
True urate	0.681
Cholesterol	0.055
Calcium	0.007
	mgm. per 100 ml.
Patient's plasma on day preceding operation:	
Total urate	7.3
Chromogen	0.7
True urate	6.6

Apart from its physiological importance as differentiating the behavior of true urate and endogenous chromogen, this finding appears possibly to have practical significance in the management of gout. Ordinarily, coffee, tea, and chocolate are forbidden to the gout patient because it is commonly supposed that they may give rise to uric acid and so increase tophaceous deposits. Actually, the methylxanthines of these beverages are metabolized to methylurates (3, 4, 14); these substances are not attacked by uricase and are therefore chromogens. Since, apparently, no chromogenic substance is deposited in the gouty tophus, it is obvious that the methylurates of exogenous origin do not contribute to tophus formation, and need not be eliminated from the diet on this account.

DISCUSSION

We may now briefly summarize our present data on the endogenous uric acid-like chromogen. This substance (or substances) is shown to be endogenous in origin since its plasma concentration and urine output are not markedly diminished by fasting. It originates spontaneously with urate in the peripheral tissue of the dog and may be formed from xanthine or hypoxanthine by the peripheral tissue of the dog. In man, who lacks uricase and appears to be comparable to the hepatectomized dog, chromogen is also formed from ingested urate precursors, as is true urate. However, injected urate also leads to chromogen production so rapidly as to suggest an enzymatic conversion. The data permit no conclusion as to whether the conversion of endogenous chromogen to urate is reversible physiologically; although it is clear that neither conversion occurs *in vitro*.

Data on a patient with leukemia and gout indicate that when urate is overproduced, chromogen is also overproduced. The chromogen clearance is of the same order of magnitude as the true urate clearance in man; and both are decreased in gout. Furthermore, the uricosuric drugs which increase urate excretion also increase chromogen excretion.

Thus, there are many resemblances between the behavior of true urate and of chromogen, but there also are some differences. Unlike true urate, chromogen is not attacked by uricase. It appears to be somewhat more freely ultrafiltrable from plasma through cellophane than is true urate ([10], Table III). It is not deposited in the gouty tophus (Table VI). There is a suggestion that, unlike urate, it is not precipitated by silver reagents (4). A critical survey of the known purine derivatives which might satisfy these criteria reveals that none appears to be satisfactory. A possible exception is 6-amino-2-8-dihydroxypurine, but so little is known of the physiology of this substance that a decision as to its importance cannot be reached.

In 1939, Ball (27) reported that the endproduct obtained when purified xanthine oxidase acted upon hypoxanthine was a substance not attacked by purified uricase, in spite of the fact that the oxygen consumption was that predicted for uric acid formation. If either crude xanthine oxidase or crude uricase were substituted for the pure enzyme in this experimental system, the reaction continued to completion.

The work of Ball is reminiscent of the much older suggestion of Gudzent (see 13) that both

[2] Through the kindness of Dr. E. F. Rosenberg.

the lactam and lactim tautomers of uric acid might be stable enough to coexist in biological fluids under certain circumstances. It is true that Ball's experiment required extremely precise conditions and, while this is undeniably an objection, it does indicate that in a suitable biological system *in vitro*, there may be a tautomer of uric acid which is not attacked by uricase. Guanase may possibly have such a selective action; this was suggested by the finding that it attacks the methylated derivatives only of the lactam tautomer of guanine (28). A more pertinent objection to identifying Ball's substance with our endogenous chromogen lies in the fact that our chromogen is not attacked by the uricase we use, which is a crude, defatted, kidney extract. At present, our feeling is that the chromogen is more likely to be a tautomer of urate than a substance such as 6-amino-2,8-dihydroxy-purine; but this is based solely on an impression that the amino substitution is unlikely from our present knowledge of the behavior of urate in the body.

Two findings appear to be of practical clinical importance. The observation that a gouty tophus contains only true urate and no chromogen, endogenous or exogenous, when considered in the light of our present knowledge that dietary methylxanthines give rise to methylurates and not to uric acid, appears possibly to indicate that the patient with gout need no longer be penalized by having coffee, tea and chocolate removed from his diet. So far as accelerating the rate of tophus deposition, these beverages appear innocuous.

The other, probably more important, conclusion bears upon the moot question of the reliability of the colorimetric estimation of urate. Our data show quite clearly that the relation between true urate and total urate in normal and gouty plasma and urine is so uniform that, if desired, true urate may be estimated from total urate values by a conversion factor. In addition, a large body of urate clearance data obtained by various investigators before the introduction of the uricase method has been shown to be almost as accurate as if true urate clearances had been studied, since the total urate clearance is almost exactly equal to the true urate clearance.

There is no question that one should use true urate determinations for maximal physiological precision, or when studying material other than human plasma and urine. However, when an estimate of the quantitative rate of nucleoprotein breakdown or turnover is desired, total urate determination appears to give a more complete picture. The chromogens discarded in the true urate determination appear to be as much a part of nucleoprotein metabolism as is true urate and, in fact, may arise from urate in the body. Only under unusual conditions, such as the ingestion of massive doses of caffeine, does the colorimetric determination on human plasma and urine become inaccurate. In contrast, the colorimetric determination of urate in human plasma or urine appears to be an entirely satisfactory method for clinical use and rather gains, than loses, in value because of a slight degree of non-specificity.

SUMMARY

Biological fluids contain material which gives the arsenophosphotungstate reaction for urate but which is not attacked by uricase. A portion of these chromogenic substances appears to be purine in nature and endogenous in origin. In many ways this endogenous uric acid-like chromogen resembles true uric acid, but it also has several differences in behavior.

The chromogen is known to be endogenous in origin since neither its plasma concentration nor urinary output is markedly affected by fasting. Unlike urate, it is not precipitated by silver, and it is somewhat more diffusible through cellophane and through the blood-brain barrier than is true urate. Increased urate production due to leukemia or induced by the feeding or injection of urate precursors leads to increases in chromogen production. This also occurs, however, when urate is injected intravenously. The chromogen clearance is of the same order of magnitude as the true urate clearance, and is similarly reduced in gout. Uricosuric drugs increase the chromogen excretion as well as true urate excretion.

For ordinary clinical pathological studies, the colorimetric determination of urate in plasma and urine, including both true urate and chromogen, appears to give a better picture of the overall rate of purine turnover than does the uricase determination. Moreover, the true urate to total urate ratio in human plasma and urine is sufficiently constant so that true urate may be reliably estimated from total urate. The true urate clearance does not differ appreciably from the total urate clearance. However, in human cerebro-

THE IMPORTANCE OF VOLUME AND OF TONICITY OF THE BODY FLUIDS IN SALT DEPLETION SHOCK [1]

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It is not known whether the deterioration in circulatory efficiency which follows sodium chloride loss is related primarily to the hypotonicity, to the changes in the volume of fluid in the various compartments, or to both. In experimental salt depletion produced by means of the intraperitoneal injection and subsequent removal of glucose solution, both of these factors invariably coexist. As sodium and chloride ions enter the intraperitoneal fluid, water moves into the cells in response to osmotic forces. Diminution of the volume of extracellular fluid and plasma, swelling of the cells, and generalized hypotonicity are the inevitable end results (1, 2, 3). Since these changes occur rapidly and almost simultaneously, it is difficult to ascribe the primary causative role in the circulatory collapse which ensues to any one factor. It is conceivable, on theoretical grounds at least, that all of these changes are important. It is quite possible, for instance, that the hypotonic and swollen cells of the cardiovascular system become inefficient, while the blood thickens and becomes more difficult to move through the vessels.

To clarify the relative significance of each these changes in the production of salt deple shock, further experiments have been cond in which hypotonicity and the state of hydra extracellular and cellular fluid have bee in directions other than those seen in o depletion.

MATERIALS AND METH

A. Dilution of the body fluids in ani- *mals.* The effects of an infusion of glucose solution on the composition of th and the efficiency of the circulation were nephrecto- mized dogs on the day followi . The second kidney. The kidneys were nembutal anaesthesia via a retroperito Two to 7 days of convalescence were ng the first operation. The glucose nistered during

a period of 1 hour in amounts sufficien lower the concentration of serum electrolytes to oximately ¾ of the initial value. After 3 to 4 hours animals received additional infusions. Two were 5 per cent saline in volumes sufficient to restore onicity of the body fluids. The third received 6 cent gelatin solution containing sodium chloride w concentrations (0.2 per cent).

B. Restoration of isoto by urea diuresis in salt- depleted animals. Two dogs were first depleted of salt by the standard te of injecting 5 per cent glucose solution intrap lly, 100 ml. per kgm., and withdrawing it aft 4 hours (1). An overnight in these animals by means of diuresis was then 350 ml. of a 15 per cent solution an intravenous i cent solution of urea in 5 per cent and 300 ml. of In one of these animals the effects glucose, resp sion of hypotonic saline, 0.6 per cent, of a subseq were inv

In all Groups A and B, movements of water llular compartment were calculated from from t chloride space (4). Alterations in the chan e were estimated from changes in the relapla ell volume and in the hemoglobin concentra- ti rial blood (1). Changes in the volume of cell were obtained by subtracting the increments or ts of extracellular water from the changes in dy water. These later values were calculated from anges in body weight; in the periods lasting 20 the weight change was corrected for water of oxi- ion according to the metabolic mixture (4). The ssibility of transfers of sodium and potassium between extracellular and cellular compartments were also investigated from the changes in their concentration in extracellular fluid, corrected for the intake and output of these ions. The flame photometer was used in these cation analyses (5). The hemodynamic studies at intervals during the course of these experiments included measurement of: (*a*) the fore-paw to medulla circulation time by means of intravenous sodium cyanide, (*b*) the mean arterial blood pressure by direct arterial puncture, and (*c*) the cardiac output by the direct Fick principle based upon the rate of oxygen consumption and the difference in the oxygen content of arterial blood from the femoral artery and mixed venous blood from the right auricle (6).

RESULTS

A. Dilution of the body fluids of nephrectomized animals. The introduction of 5 per cent glucose

[1] Aided by a grant esearch Fund of Yale University.

[2] Dr. Winkler di

TABLE I

Exchanges of water, electrolytes, and nitrogen

Experiment	Procedure	Time after start of experiment	Weight*	Intake					Output									Balance†	
				Intravenous				i.p.	Peritoneal or G. I. fluid				Urine						
				H_2O	Cl	Na	N	H_2O	Vol.	Cl	Na	K	Vol.	Cl	Na	K	N	Cl	K
		hours	kgm.	ml.	meq.	meq.	grams	ml.	ml.	meq.	meq.	meq.	ml.	meq.	meq.	meq.	grams	meq.	meq.
A. Dilution of body fluids in nephrectomized animals with normal salt content																			
110B		0	8.82																
	5 per cent glucose i.v.	2.5**	9.83	1250					100‡	10.2	5.3	—						− 12	± 0
		4.5	9.65															− 1	± 0
	5 per cent NaCl i.v.	7.3	9.83	250	213	213												+211	± 0
113		0	8.15																
	5 per cent glucose i.v.	3.5	9.26	1100					30‡	3.2	1.8	—						− 6	± 0
	5 per cent NaCl i.v.	5.7	9.22	200	171	171			80§	7.3	9.1	—						+160	± 0
115		0	10.20																
	5 per cent glucose i.v.	2.7**	11.28	1250					120‡	16.2	7.3	1.4						− 19	− 1
	6 per cent gelatin i.v.††	5.2	11.54	300	4.8	4.8	—											+ 3	± 0
B. Salt depletion and restoration of isotonicity by urea diuresis																			
111		0	9.10																
	5 per cent glucose i.p.‡‡	4	9.08					1000	940‡‡	70.6	82.7	2.4	0					− 72	− 2
	15 per cent urea i.v.§§	25.5	8.25	350			25.2						710	2.4	2.1	12.8	13.4	− 6	−13
	6.7 per cent NaCl i.v.	28.5	8.60	800	92	92							360	7.1	11.1	10.5	6.1	+ 83	−11
116		0	7.85																
	5 per cent glucose i.p.‡‡	4.3	7.74					800	830‡‡	59.3	75.7	1.9	0					− 61	− 2
	10 per cent urea i.v.§§	23	7.02	300			14.0						500	4.6	3.2	7.1	7.7	− 6	− 7

* Weight at end of period is corrected for solids lost as feces and red cells taken for analysis.
† See Table II for Na balance; balances are corrected for small amounts of Cl and Na lost in serum taken for analysis.
‡ Vomitus.
§ Diarrheal fluid.
** Convulsions of water intoxication.
†† Gelatin with low salt content obtained from the Knox Gelatine Co.
‡‡ Peritoneal fluid withdrawn at end of period.
§§ Urea given in 5 per cent glucose solution.
In both tables, balance data are expressed per individual period rather than cumulatively. Time from start of experiment indicates end of period at which time serum analyses and hemodynamic measurements were made.

TABLE II

Analytical data, hemodynamic measurements, and changes in body fluids

Experiment	Procedure	Time after start of experiment	Serum*			Blood						Oxygen		Cardiac index	Change in						
			Cl	Na	K	Total protein	Relative cell volume	Hemoglobin	NPN	Circulation time	Mean arterial pressure	Consumption	A-V difference		Plasma volume	Total water	Extracellular fluid	Intracellular fluid	Total body Na	Total ionic conc.†	Total osmotically active base
		hours	*meq. per liter*	*meq. per liter*	*meq. per liter*	*grams per cent*	*per cent*	*grams per cent*	*mgm. per cent*	*seconds*	*mm. Hg*	*ml. per minute*	*vol. per cent*	*liters per min. per sq. meter*	*ml.*	*liters*	*liters*	*liters*	*meq.*	*meq. per liter*	*meq.*
	Dilution of body fluids in nephrectomized animals with normal salt content																				
110B	5 per cent glucose i.v.	0	93.4	154.0	5.6	6.52	44.7	15.7	91	9	177	89	7.3	2.84	− 5	+1.01	+0.70	+0.31	− 8	−43	−132
		2.5	68.5	113.8	5.0	4.69	43.5	16.2	—	9	177	97	3.0	7.52	− 12	−0.18	−0.32	+0.14	− 2	+10	+ 50
		4.5	76.3	123.8	4.2	5.03	45.8	15.9	—	10	162				+454	+0.18	+0.62	−0.44	+211	+29	+ 22
	5 per cent NaCl i.v.	7.3	122.8	155.1	4.4	3.13	28.3	10.7	86	9	170										
113	5 per cent glucose i.v.	0	103.0	142.9	5.5	5.16	34.3	11.4	83	9	90	93	10.2	2.28	+189	+1.11	+0.62	+0.49	− 6	−31	− 34
	5 per cent NaCl i.v.	3.5	78.0	113.6	4.4	3.71	26.4	9.0	—	10	87	84	5.9	3.53	+230	−0.04	+0.79	−0.83	+158	+32	+ 53
		5.7	101.2	146.6	4.4	3.05	20.0	7.2	83	11	74	91	4.2	5.40							
115	5 per cent glucose i.v.	0	104.3	150.2	5.4	6.87	44.3	14.6	107	8	106	95	5.[illegible]	3.62	+236	+1.08	+1.18	−0.10	− 11	−38	−124
	6 per cent gelatin i.v.††	2.7	68.3	114.5	3.2	5.57	37.5	12.8	119	8	106	91	2.[illegible]	6.68	+286	+0.26	−0.07	+0.33	+ 2	+ 8	+ 98
		5.2	70.5	122.4	3.1	‡	29.1	9.9	‡	6	132	71	3.[illegible]	5.05							
	Salt depletion and restoration of isotonicity by urea diuresis																				
114	5 per cent glucose i.p.	0	108.2	155.3	4.7	6.37	43.8	14.8	37	7	118	83	8.2	2.35	−158	−0.02	−0.30	+0.28	− 85	−20	− 45
	15 per cent urea i.v.§§	4	90.7	133.0	4.2	8.72	55.0	17.3	—	11	92	83	8.5	2.28	+ 28	−0.70	−0.37	−0.33	− 7	+24	+ 52
	6.7 per cent NaCl i.v.	25.5	106.9	154.8	5.6	9.75	50.5	17.6	315	13	84	79	4.2	1.37	+175	+0.35	+0.44	−0.09	+ 78	+ 1	− 1
		28.5	122.2	160.2	3.5	6.87	42.0	14.0	148	9	90										
116	5 per cent glucose i.p.	0	99.5	146.8	4.1	5.59	53.5	17.0	31	7	150	83	10.4	2.05	−138	−0.11	−0.31	+0.20	− 78	−14	− 13
	10 per cent urea i.v.§§	4.3	84.6	130.5	4.3	7.50	62.8	20.0	39	12	120	62	9.7	1.64	+ 45	−0.64	−0.24	−0.40	− 5	+ 7	− 48
		23	94.3	137.2	7.0	7.76	58.7	19.3	240	12+	50										

* Water content of serum (W_s) calculated from the serum total protein concentration (P_s) by the formula (10): $W_s = 99.3 - 0.889\ P_s$.

† Total ionic concentration was assumed to equal the concentration of Na in extracellular water + 10 milliequivalents per liter (4).

‡ [illegible] total protein and NPN concentrations were not determined because of the presence of gelatin; water content of serum was assumed to be unchanged.

[illegible] Table I.

solution produced hypotonicity, an expansion of the extracellular fluid volume in all experiments, as well as an increase in the water of tissue cells in 2 of the 3 animals (Tables I and II). Despite this marked distortion in the concentration of the electrolytes and a swelling of tissue cells quite as great as that witnessed in salt depletion, circulatory efficiency was not adversely affected. The mean arterial pressure and the circulation time were unaltered. The cardiac output was moderately depressed prior to the dilution of body fluids in the 2 experiments in which initial observations are available. This was presumably related to the recent nephrectomy. It is highly significant, however, that it did not decline further following the intravenous glucose solution. As a matter of fact, in one of the dogs (Experiment 115), the cardiac output rose to a level that was well above normal.[3] These findings are in direct contrast to the profound deterioration of the circulation which accompanies salt depletion (1).

The subsequent restoration of a normal concentration of serum electrolytes in Experiments 110B and 113 by means of hypertonic saline resulted in a normal and a supranormal cardiac output, respectively, in contrast to the previous moderately depressed values. Expansion of the plasma volume without restoration of tonicity in Experiment 115 had no adverse effect on the already high normal level of cardiac output.

B. Restoration of isotonicity by urea diuresis in salt-depleted animals. It is evident from Tables I and II that even though the hypotonicity of the body fluids and the swelling of the cells were partially or fully corrected by this dehydrating procedure, circulatory dynamics either failed to improve or actually deteriorated. The volume of extracellular fluid declined further in both experiments. Replacement of the salt deficit and reexpansion of the extracellular fluid by means of saline partially restored the cardiovascular function which had not been improved by the correction of the hypotonicity (Experiment 114).

In none of the experiments in this group nor in the nephrectomized animals was any significant exchange of extracellular sodium for cell potassium detected. In the nephrectomized animals a large amount of intracellular base was apparently inactivated following the glucose infusion (Table II) (7). This was reflected in the disproportionately large volume of the administered fluid which remained in the extracellular phase. The alternative explanation is that a water gradient was present which decreased between 2.5 and 4.5 hours (Experiment 110B). It is hard to believe that such a long period was necessary for the distribution of water to equilibrium. The unequal phase of distribution could also be accounted for by the entry of chloride into cells; such a phenomenon, however, would in no way affect the calculation of exchanges of total water and total osmotically active base. Following the administration of hypertonic saline or of low salt colloid, this base once again became osmotically active.

DISCUSSION

For purposes of discussion published data on simple dehydration and on salt depletion studies are summarized in Figure 1 for comparison with the dilution experiments in the nephrectomized animals. Analysis of these 3 sets of experiments permits certain generalizations. It is immediately apparent that an expansion of the body water, as in the dilution studies with nephrectomized dogs, does not interfere with circulatory efficiency (Column A). A decrease in the total body water, on the other hand, such as that which occurs in simple dehydration does impair cardiovascular function to a limited degree (Column B). This is evident in the drop in the cardiac output, but the decrease is by no means marked or consistent. In the third group of experiments, those in which salt depletion was produced, profound deterioration of the circulatory efficiency was present (Column C). In these animals the total amount of body water remained essentially unchanged. It is apparent, therefore, that extensive alterations in the total amount of body water can be induced with only moderate, if any, effect on cardiovascular dynamics, and that marked circulatory inefficiency can develop even though the total volume of water in the body remains constant. These findings suggest that factors other than the total body water condition cardiovascular function.

Study of the changes which occur in the various subdivisions of the total body water in these 3

[3] Mean cardiac index in normal dogs was found by the authors to be 5.45 ± 1.43 liters per minute per square meter (1).

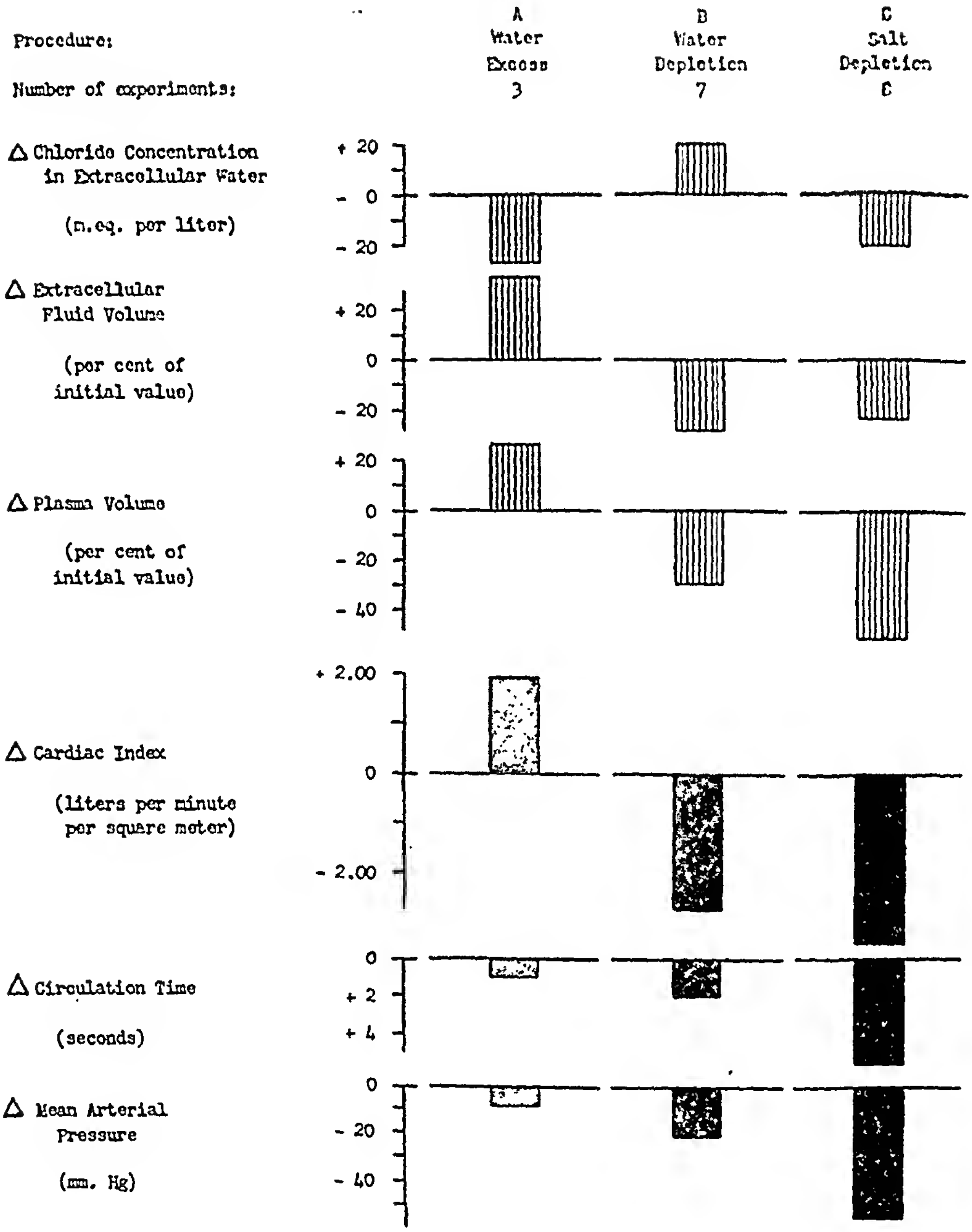

FIG. 1. CHANGES IN CIRCULATORY DYNAMICS FOLLOWING VARIOUS ALTERATIONS IN THE BODY FLUIDS

Each column represents the mean value for the number of experiments indicated. Columns B and C are taken from previously reported data (1). The changes shown were produced in animals with normal body fluids. It is evident that a decrease in both volume and concentration (salt depletion experiments in Column C) causes more profound circulatory deterioration than does a similar change in either volume alone (water depletion experiments in Column B) or in concentration alone (water excess experiments in Column A).

groups of experiments serves to identify some of these factors. In the dilution experiments with the nephrectomized preparations, cell water, extracellular water, and plasma water were all overexpanded without circulatory impairment. In the simple dehydration experiments all of these compartments were contracted in volume with some attendant deterioration of the circulation. In the third group of animals, those depleted of salt, only the extracellular water and the plasma water were decreased, and yet profound shock was present. The decrease in the plasma volume, however, was much greater in the salt-depleted dogs than in the simple dehydrated animals. It would appear, there-

fore, from these experiments, as it has from work on other types of shock, that the volume of plasma is an important factor in the development or manifestations of the salt depletion shock state. The increase in the volume of cell water which attends salt depletion is probably not a contributing factor, since similar degrees of cellular overhydration in the diluted nephrectomized dogs had no deleterious effect on the circulation.

Up to this point no account has been taken of the changes in tonicity which accompany these manipulations of the water in the various compartments of the body. The body fluids of the dehydrated animals were hypertonic, while the salt-

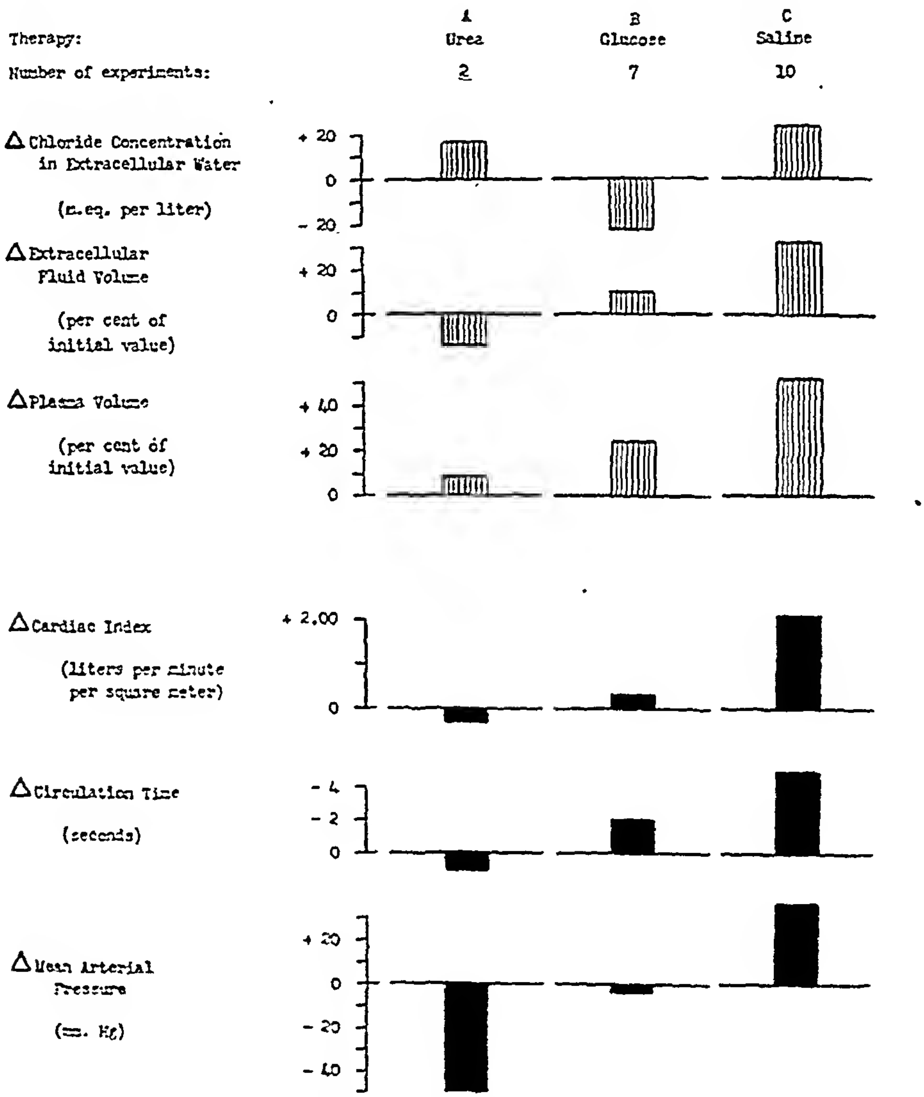

FIG. 2. CHANGES IN CIRCULATORY DYNAMICS FOLLOWING VARIOUS TYPES OF TREATMENT OF SALT DEPLETION SHOCK

Each column represents the mean value for the number of experiments indicated. Columns B and C are taken from previously reported data (2). The changes shown were produced in animals already in salt depletion shock. In such a state, restoration of concentration alone by urea diuresis (Column A), or of volume alone by glucose infusion (Column B), failed to improve the circulation. Restitution of both volume and concentration by infusion of saline (Column C) was followed by prompt recovery from the shock state.

depleted dogs became hypotonic. Since hypertonicity was accompanied by less striking changes in the circulation, hypotonicity, as seen in salt depletion, would appear to have a significantly greater adverse effect on cardiovascular function. Hypotonicity, however, cannot be the sole determinant, since a comparable lowering of the concentration of body electrolytes was produced in the dilution experiments with nephrectomized dogs without impairment of the circulation. In these animals, however, in contrast to those depleted of salt, the volume of body water was expanded rather than contracted.

These facts suggest, therefore, that not only the volume of fluid in the body and its various compartments, but also the concentration of electrolytes present are important determinants of whether or not salt depletion shock develops.

The statement that both the volume of extracellular fluid and plasma and the concentration of electrolytes in the body fluids operate in maintaining the integrity of the circulation can be further supported by analysis of the responses of salt depletion shock to various forms of treatment. From Figure 2 it is immediately evident that restoring concentration alone, as in the urea diuresis experiments (Column A), or restoring volume alone as in the animals given glucose solution (Column B), failed to relieve the circulatory collapse produced by salt depletion. Yet when both volume and concentration were restored by saline infusions (Column C), prompt recovery from shock was observed.

The finding that both the volume of body fluids, especially plasma, and the concentration of electrolytes in them are important factors in maintaining circulatory efficiency has many clinical implications. The most obvious of these lies in the well-known fact that extensive depletion of body water and electrolytes, as in patients who sweat or who have losses of gastrointestinal fluid, can coexist with isotonicity. These normal concentrations, however, can no longer be viewed with the assurance that they protect the organism against salt depletion shock, nor can they be interpreted as evidence against a need for salt and water. This is true even though isotonicity is encountered only in the less severe degrees of salt depletion. Nonetheless, in these patients as well as in those with greater salt deficits, when volume is restored tonicity should be maintained. If salt solutions alone do not restore cardiovascular efficiency, supportive treatment with colloid solutions is indicated.

It should be pointed out, however, that hypotonicity is not necessarily the result of a loss of salt in excess of water. It can also indicate simple dilution, as in anuric or oliguric patients treated with excessive amounts of non-electrolyte containing fluids. The problem becomes even more complicated when both depletion and dilution are present in the same subject, as in nephritics and patients on a limited intake of salt during periods of salt loss. Irrespective of whether the hypotonicity is the result of depletion or dilution, the patient should receive treatment which restores the concentration to normal. Withholding salt in such patients because of the fear of augmenting edema or congestive heart failure may prove to be a serious error, whereas the circumspect use of saline almost always turns out to be either beneficial or benign (8, 9).

SUMMARY AND CONCLUSIONS

1. Hypotonicity of body fluids produced by glucose infusions in nephrectomized dogs with intact body salt stores did not impair circulatory efficiency.

2. Elimination of hypotonicity and cellular overhydration following urea diuresis in dogs in salt depletion shock, without replacement of salt deficits and extracellular fluid reexpansion, failed to improve cardiovascular dynamics.

3. Both the volume of fluid in the body, particularly the volume of plasma, and the tonicity of this fluid are important factors in salt depletion shock.

ACKNOWLEDGMENT

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BIBLIOGRAPHY

1. Elkinton, J. R., Danowski, T. S., and Winkler, A. W., Hemodynamic changes in salt depletion and in dehydration. J. Clin. Invest., 1946, 25, 120.
2. Danowski, T. S., Winkler, A. W., and Elkinton, J. R., The treatment of shock due to salt depletion; comparison of the hemodynamic effects of isotonic saline, of hypertonic saline, and of isotonic glucose solutions. J. Clin. Invest., 1946, 25, 130.

3. Winkler, A. W., Danowski, T. S., and Elkinton, J. R., The role of colloid and of saline in the treatment of shock. J. Clin. Invest., 1946, 25, 220.
4. Elkinton, J. R., and Winkler, A. W., Transfers of intracellular potassium in experimental dehydration. J. Clin. Invest., 1944, 23, 93.
5. Hald, P. M., The flame photometer for measurement of sodium and potassium in biological materials. J. Biol. Chem., 1947, 167, 499.
6. Grollman, A., The Cardiac Output of Man in Health and Disease. C. C. Thomas, Springfield, Illinois, 1932.
7. Elkinton, J. R., Winkler, A. W., and Danowski, T. S., Inactive cell base and the measurement of changes in cell water. Yale J. Biol. & Med., 1944, 17, 383.
8. Elkinton, J. R., Danowski, T. S., and Winkler, A. W., The clinical interpretation of changes in the concentration of chloride in serum. Unpublished.
9. Danowski, T. S., Winkler, A. W., and Peters, J. P., Treatment of oliguria and anuria. Unpublished.
10. Danowski, T. S., Elkinton, J. R., and Winkler, A. W., The deleterious effect in dogs of a dry protein ration. J. Clin. Invest., 1944, 23, 816.

THE EFFECT OF INCREASED INTRA-ABDOMINAL PRESSURE ON RENAL FUNCTION IN MAN[1]

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It is well established that increased intra-abdominal pressure alters renal function. In experimental animals, distention of the abdominal cavity under pressures greater than 15 mm. of mercury usually reduces urine flow and, under pressures greater than 30 mm., stops urine flow altogether (1). In man, a tense accumulation of ascitic fluid may be associated with oliguria and the retention of water and salt, which disappear following paracentesis (1). In one respect, increased intra-abdominal pressure seems to improve the function of the kidney, for the urine may become highly concentrated during the period of oliguria. This effect has found practical application in the hands of radiologists (2, 3), who use abdominal compression during intravenous pyelography to assure maximum density of the contrast media excreted by the kidneys.

Until the present there has been no satisfactory explanation for these alterations in renal function during abdominal pressurization. It has been suggested that they are due to partial urinary obstruction (3), but the radiologic evidence is against this explanation (4). Urine flow may be increased by diuretics during the application of pressure in experimental animals, indicating the absence of obstruction. Thorington and Schmidt (1) have called attention to the possibility that the associated increased renal venous pressure may interfere with normal blood flow through the kidney and disturb renal function.

Recently, abdominal compression has been used as a means of protecting airmen against the ill-effects of centrifugal force (positive G) (5). It was assumed that pressurization prevents the pooling of venous blood in the abdominal veins and sustains the return of blood to the heart. The present study, undertaken primarily to determine whether such procedures have detrimental effects on the abdominal organs (6), revealed that the diversion of arterial blood from large visceral circuits to more vital regions, such as the brain, is also an important factor.

METHODS

The clearance and saturation methods devised by Smith and his coworkers (7 to 9) were used to evaluate renal hemodynamics and tubular activity during abdominal compression in 17 normal human subjects, ranging in age from 17 to 50, almost equally divided as to sex, and clinically free of cardiovascular and renal disease. The effective renal plasma flow was measured by the diodrast or p-aminohippurate (PAH) clearances; and the glomerular filtration rate, by the mannitol or inulin clearances.[2] The maximal rate of tubular glucose reabsorption (glucose Tm) and the maximal tubular diodrast excretory rate (diodrast Tm) were determined to estimate tubular function and to assess the distribution of filtrate and perfusate to tubular tissue.

The pressure within the abdomen was increased by inflating a rubber bladder with air under pressure, beneath a girdle tightly applied to the abdomen. The bladder, sewn into a pocket in the girdle, covered the anterior abdominal wall. Pressures between 70 and 80 mm. Hg were applied during the determination of clearance and Tm values. These pressures were usually endured by the subject without complaint. Occasionally, however, slightly lower pressures were required or the period of application was shortened because of discomfort and respiratory difficulty. Among the subjects of the studies reported here, only very slight changes in blood pressure were observed during pressurization.

All subjects were examined in the basal fasting state, resting quietly in bed. Following the establishment of appropriate plasma levels of the test substances by a

[1] This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts Memorial Hospitals.

[2] A supply of sterile solutions of sodium p-aminohippurate (20 per cent) and mannitol (25 per cent) was made available for use in this study through the courtesy of the Medical Research Division of Sharpe and Dohme, Incorporated, Philadelphia, Pennsylvania. Diodrast was obtained in sterile solution (35 per cent) from the Winthrop Chemical Company, New York City; and inulin (10 per cent) from William R. Warner and Company, New York City.

technique described at length elsewhere (7), the bladder was catheterized and washed out with a measured volume of isotonic saline solution. Urine was then collected by catheter at intervals of 10 to 15 minutes and carefully measured. After 2 or more control periods had been obtained, abdominal compression was continuously applied for 10 to 45 minutes and 1 to 3 collections of urine were made during this time. On most occasions, 2 or more urine-periods were obtained after the pressure was released. Samples of venous or arterial blood were taken at 30-minute intervals.

Diodrast, PAH, mannitol, and inulin were determined in cadmium filtrates of plasma (10) and in dilute samples of urine, using methods described by Goldring and Chasis (8). Glucose was determined by the Nelson-Somogyi method (11) in cadmium filtrates of plasma and diluted urine. Clearance and Tm values were calculated by the procedures of Smith (7, 8).

In another group, comprising 15 subjects, a catheter was placed in the renal vein and venous pressures measured during the application of increased abdominal pressure. An extension of the technique developed by Cournand and Ranges (12) for the catheterization of the right atrium was employed. A small incision was made over a median basilic vein under local anesthesia and the vein secured by a suspensory ligature. A small longitudinal incision was then made in the vein with a bayonet scalpel, and an extra-length ureteral catheter [3] with a curved tip (Cournand tip) was introduced into the lumen of the vein. Sterile isotonic saline solution flowed constantly through the catheter at a slow rate throughout the procedure to prevent blockage by clotting. The catheter was next passed into the superior mediastinum under fluoroscopic control and then into the chamber of the right atrium. The orifice of the inferior vena cava was entered with difficulty on many occasions, apparently owing to obstruction by the valve of the inferior vena cava. This obstruction could be passed by rotating the tip of the catheter to the right and posteriorly during passage through the atrium. In the inferior vena cava, the tip was directed to the right and usually passed with little difficulty into the right renal vein. The catheter tip could be localized far out to the right side of the abdominal cavity below the shadow of the liver. The left renal vein was catheterized less frequently and with somewhat greater difficulty. Pressure determinations were made in the renal vein and in the upper portion of the inferior vena cava with a saline manometer after the method of Moritz and von Tabora (13).

In 8 subjects, catheterization of the right renal vein was carried out for the determination of the percentage extraction of PAH by the kidney during abdominal pressurization. In this procedure, a constant plasma level of PAH (1 to 2 mgm. per cent) was established. Samples of blood were obtained simultaneously from the right renal vein and from a peripheral vein in the arm opposite to that in which the infusion was administered. The extraction percentage was calculated by dividing the difference between the concentration of PAH in the peripheral and renal venous blood by its concentration in the peripheral blood. This value, the percentage of PAH removed from each milliliter of blood as it traverses the kidney, was determined before, during, and following application of abdominal pressure.

RESULTS

A. Alterations in renal blood flow and glomerular filtration during abdominal compression

1. *Intra-abdominal pressure and renal venous pressure*

When the rubber bladder of the abdominal pressure cuff was inflated with air at pressures between 70 and 80 mm. Hg, it was found that intra-abdominal pressure measured at various locations within the abdomen averaged 20 mm. Hg, within a very small range. This value was determined with a saline or Hamilton manometer in the stomach, duodenum, and rectum by means of the Miller-Abbott tube (6), and in the bladder and renal pelvis by catheters. Such a uniform elevation of pressure throughout the confines of the abdomen provided evidence in support of the view that the abdomen and its contents could be considered as relatively non-compressible and fluid in character, behaving in accordance with Pascal's law. The difference in pressure between the cuff and the peritoneal cavity probably resulted from the resistance to compression by the abdominal wall and from the resistance to extension of pressure by the diaphragm.

The pressure in the cuff represented, therefore, the average force exerted upon the anterior abdominal wall, and did not necessarily reflect the pressure within the abdominal cavity. As a matter of fact, the intra-abdominal pressure varied considerably depending upon the state of contraction of the abdominal musculature. Thus, the average figure of 20 mm. Hg was typical of complete relaxation, whereas pressures as high as 176 mm. Hg (Table I) were recorded from the renal vein during a strong "bearing down" contraction of abdominal muscles. Contrariwise, increased rigidity of the abdominal wall, in pushing away the inflated bladder, likely altered the transmission of

[3] The catheters used in this study were manufactured by the United States Catheter and Instrument Corporation, Glens Falls, New York. They are 100 centimeters long and terminate in a curved tip (Cournand tip) with 1 eye.

TABLE I

Intra-abdominal venous pressure before, during, and after abdominal compression

Pressure applied to anterior abdominal wall by pneumatic girdle at 80 mm. Hg. Venous pressure was measured with a saline manometer before, during, and after (recovery) the application of pressure. The pressures tabulated under "Strain" were obtained when the subject voluntarily increased his intra-abdominal pressure by a "bearing-down" maneuver, during the application of pressure.

Subject	Control	Abdominal compression	Strain	Recovery
Renal vein				
	mm. Hg	*mm. Hg*	*mm. Hg*	*mm. Hg*
C. M.	5.8	18.1	42.6	5.8
R. S.	6.4	20.2	45.8	9.6
J. S.	6.2	17.6	75.6	—
W. C.	7.9	20.2	88.1	—
D. C.	7.0	22.1	108.0	5.7
O. C.	5.3	14.1	—	—
J. F.	5.1	18.9	176.0	—
H. B.	2.9	17.8	—	—
M. B.	5.8	16.0	101.0	8.2
Inferior vena cava				
C. W.	5.2	13.6	44.1	—
O. C.	2.4	24.5	38.1	—

cuff pressure into the abdominal cavity. In certain well-muscled individuals (J. Q., Table II) the absence of renal functional change during abdominal compression was ascribed to this factor.

Owing to the technical difficulties involved in measuring and maintaining constant intra-abdominal pressures, a constant compressional pressure was used during these studies, with the assumption that the averaged results could be discussed in terms of an average intra-abdominal pressure of 20 mm. Hg. This proceeding seemed justified since the voluntary effort of resisting the cuff was fatiguing and not usually sustained. Most subjects quickly adjusted themselves to the compression and relaxed.

Renal venous pressure partook in the generalized rise of intra-abdominal pressure. In 9 subjects (Table I) the control renal venous pressure of 5.8 mm. Hg increased to 18.3 mm. on the average. A similar increase of pressure in the upper portion of the inferior vena cava was observed twice (C. W. and O. C., Table I). It is probable that mean arterial pressure within the abdomen was not altered since extra-arterial pressures lower than diastolic pressure cannot affect intra-arterial mean pressure (14). Hence, the head of pressure at the beginning of the renal circuit probably did not rise during compression while the venous pressure increased 3- to 4-fold. Such a change in the gradient of pressure along the renal vascular tree might be expected to result in a reduction of renal blood flow. This expectation was borne out by clearance studies.

2. *The effective renal blood flow*

Figure 1 illustrates the renal hemodynamic response to abdominal compression. In this subject (J. S.), the effective renal plasma flow (sodium p-aminohippurate clearance, C_{PAH}) remained relatively unchanged during the control periods, but fell strikingly from an average of 827 ml. per minute to 270 ml. per minute during pres-

TABLE II

Effect of increased intra-abdominal pressure on renal plasma flow and glomerular filtration rate

All figures, with the exception of those marked with an asterisk, are averages of 2 or more periods.

Subject	Sex	Age	Control: Glomerular filtration rate	Control: Effective renal plasma flow	Control: Filtration fraction	During pressure: Glomerular filtration rate	During pressure: Effective renal plasma flow	During pressure: Filtration fraction	Recovery: Glomerular filtration rate	Recovery: Effective renal plasma flow	Recovery: Filtration fraction
			ml. per min.	*ml. per min.*	*per cent*	*ml. per min.*	*ml. per min.*	*per cent*	*ml. per min.*	*ml. per min.*	*per cent*
H. B.	Male	21	102.0	827	12.3	*67.6	640	10.6	*98.5	764	13.0
P. K.	Male	43	113.0	536	21.0	*83.5	432	19.3	*139.7	686	20.4
A. N.	Female	45	128.0	655	19.5	120.7	581	20.8	112.0	559	20.1
A. C.	Female	31	87.5	541	17.8	81.8	437	18.7	83.0	388	22.1
N. P.	Female	27	94.8	625	15.1	66.7	437	15.3	100.7	656	15.4
J. S.	Female	26	125.3	827	15.2	43.8	269	16.2	117.5	697	16.8
M. E.	Female	43	122.7	540	22.7	107.0	399	27.7	157.0	486	32.2
J. Q.	Male	42	143.4	834	17.6	143.7	884	16.3	153.1	966	15.9

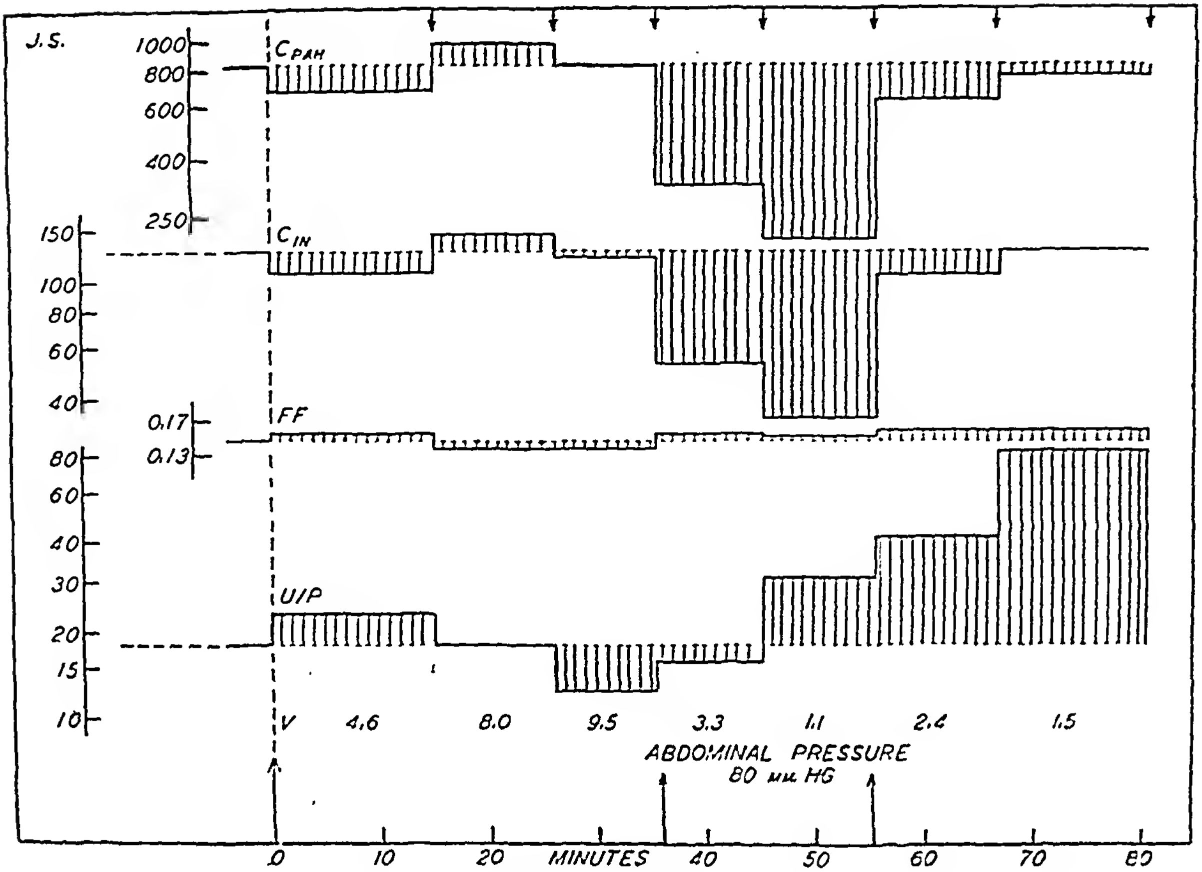

FIG. 1. ALTERATIONS IN EFFECTIVE RENAL PLASMA FLOW AND GLOMERULAR FILTRATION RATE DURING ABDOMINAL COMPRESSION IN SUBJECT J. S.

The effective renal plasma flow (ml. per min.) measured by the p-aminohippurate clearance (C_{PAH}) and the glomerular filtration rate (ml. per min.) measured by the inulin clearance (C_{IN}) were determined during the application of 80 mm. Hg pressure to the abdominal wall. The filtration fraction (FF), the percentage of plasma filtered (C_{IN}/C_{PAH}), remained unchanged throughout. During compression the concentration ratio of inulin (U/P) increased and continued to rise after the pressure was released. Urine flow (V) (ml. per min.) fell markedly. Arrows at the top of the chart indicate the time of urine collection.

surization. There was a rapid return to the control level following the release of pressure.

Likewise, in 7 of the 8 subjects (Table II) in which it was determined, the effective renal plasma flow was reduced, although the *average* reduction was not so large in the other subjects as in J. S. In several individuals, during 1 or more periods immediately after the application of the abdominal cuff, the reduction in effective renal plasma flow was as large as that seen in J. S., but these changes are minimized in the calculation of the average figure by the inclusion of periods in which evidence of circulatory readjustment was observed. In these subjects, where the time of observation was sufficiently prolonged (more than 30 minutes of compression), the effective renal plasma flow and other renal functions (4 of 10 studies) showed a return toward the control values despite continued compression.[4] Nevertheless, when all the figures were averaged disregarding this fact and including J. Q., in whom abdominal compression produced no change (possibly because of abdominal muscular resistance), it was found that effective renal plasma flow was reduced from 673 ml. per minute to 509 ml. per minute, an average reduction of 24.4 per cent.

[4] Figure 2 (J. W.) illustrates [illegible] of the maximal tubular [illegible] excretory rate [illegible] and the glomerular filtration rate [illegible] plasma [illegible]

It is noteworthy that renal hyperemia did not occur following the period of compressional ischemia. Occasionally very short urine periods collected immediately after release of pressure yielded high clearance values. These anomalous values may be attributed to a relatively larger contribution of dead-space urine to the total. Usually, however, high clearance values during recovery were absent. Indeed, the averaged figures of Table II reveal that effective renal plasma flow during recovery tended to be lower than during the control period. The absence of reactive hyperemia in the kidney observed during this study is in agreement with a large body of evidence obtained by others in the study of the renal circulation in man and other mammals (15, 16).

Proteinuria. It has been repeatedly demonstrated that a reduction of renal blood flow following obstruction to renal venous drainage is associated with the appearance of proteinuria (17). With abdominal pressure also, it was found that protein appeared in the urine in variable amounts. In 1 subject who had no history of previous renal disease and who was in good health, a 2 + proteinuria developed during the application of the abdominal cuff. On most occasions, however, the proteinuria was not striking, although it occurred regularly.

Validity of the method of renal blood flow measurement. It is possible that these changes in clearance values were caused by errors inherent in the method of measurement rather than by alterations of renal plasma flow. If pressure reduced the capacity of renal tubular cells to remove PAH from the blood, the clearance value would fall because of incomplete clearance of PAH. Or, if blood were shunted away from tubular tissue into the veins so that an opportunity for complete clearance were lacking, the clearance value might be decreased despite an absence of change (or even an increase) in blood flow.

The determination of renal PAH extraction, using the catheterization technique, provided the answer to this problem. The data summarized in Table III reveal that no change in PAH extraction occurred during compression. Hence, the clearance of PAH and, by analogy, of diodrast, provides an accurate measure of the behavior of renal plasma flow during increased abdominal pressure. In addition, the possibility that arteriovenous shunting occurs during increased pressure is excluded by this evidence.

TABLE III

Renal extraction of sodium p-aminohippurate before, during, and after abdominal compression

Subject	Control	Abdominal compression	Recovery
	per cent	*per cent*	*per cent*
C. M.	90.0	93.6	93.8
D. C.	91.0	93.2	91.9
S. G.	92.8	94.0	93.5
O. C.	94.2	90.7	—
M. B.	93.8	95.3	95.8
F. H.	90.5	91.5	92.5
M. H.	92.1	92.3	—
L. J.	94.5	93.2	91.6

Storage of PAH or diodrast during compression by nephrons that continue to be capable of excretory activity although unable to contribute to urine formation, might also introduce an error in the clearance determination that would elude detection in the measurement of the extraction ratio. However, this phenomenon would necessarily result in falsely high clearance values during recovery as a result of storage wash-out, and the relationship between calculated filtration rate and effective renal plasma flow would be thus altered artificially. This effect was never observed.

It appears that the clearance technique may be used with confidence under these conditions for the measurement of effective renal plasma flow, since extraction of PAH was not altered and storage probably did not occur.

3. *Glomerular filtration rate and filtration fraction*

The glomerular filtration rate was nearly always greatly reduced. Among the group collated in Table II, filtration fell to 88.4 ml. per minute (on the average) from the control level of 117.2 ml. per minute, a 27.5 per cent decrease. This observation was repeatedly confirmed in other studies in which renal plasma flow was not measured (see Table IV). Of particular significance is the fact that glomerular filtration was reduced to the same extent as the effective renal plasma flow. This equal percentile reduction was not a statistical accident but was observed whenever renal plasma flow decreased, as in Figure 1. Consequently, the ratio between filtration and plasma flow, the filtra-

TABLE IV

Effect of increased intra-abdominal pressure on urine flow and urinary concentration

The urinary concentration is expressed as the concentration ratio of inulin or mannitol calculated by dividing the urinary concentration by the plasma concentration. All U/P ratios are averages of 2 or more periods with the exception of those obtained during compression. These values are the maximal concentrations observed and they are presented with figures for filtration rate and urine flow determined simultaneously. All other values, except those marked with an asterisk, are averages of 2 or more periods.

Subject	Control			Abdominal pressure			Recovery		
	Glomerular filtration rate	Urinary concentration	Urine flow	Glomerular filtration rate	Urinary concentration	Urine flow	Glomerular filtration rate	Urinary concentration	Urine flow
	ml. per min.		*ml. per min.*	*ml. per min.*		*ml. per min.*	*ml. per min.*		*ml. per min.*
P. K.	127.5*	40.0*	3.1*	83.5	39.0	2.4	139.7*	38.3*	3.6*
A. N.	128.0	29.0	4.4	116.3	38.1	3.1	113.0	37.0	3.0*
A. C.	87.2	25.1	3.5	80.0	28.2	2.8	86.0	28.0	3.0
N. P.	94.8	27.5	3.5	43.7	27.1	1.6	100.0	32.1	3.1
S. Q.	143.0	17.6	8.2	144.0	24.9	5.7	153.1	22.9	6.7
M. S.	97.5	27.4	3.5	79.0	36.6	2.2	115.3	28.8	3.9
J. Si.	155.0	16.9	9.2	110.0	26.5	4.2	193.9	21.9	8.8
L. R.	126.8	11.9	10.9	112.7	34.7	3.2	126.8	29.3	4.3
M. P.	114.9	11.3	10.0	67.4	20.0	3.4	105.4	16.1	6.7
L. R.	98.8	8.4	11.8	57.7	24.5	2.4	112.5*	21.2*	5.3*
H. B.	102.0	24.8	4.1	67.6	33.2	2.0	98.5*	32.8*	3.0*
J. S.	125.3	18.2	7.4	34.3	23.5	1.1	117.5	64.7	1.9
J. W.	123.9	6.7	18.4	80.9	40.5	2.0	144.7	58.6	2.4
M. E.	122.7	8.5	12.1	108.8	34.4	3.3	157.0	31.1	5.0
L. G.	111.0	12.8	9.3	79.6	15.9	5.0	120.2*	15.8*	7.6*
H. B.	98.0	28.1	3.5	72.0	31.3	2.3	78.5	32.0	2.5
J. Q.	143.4	17.6	8.2	144.0	25.0	5.8	153.1	22.9	6.7

tion fraction, or the percentage of plasma filtered at the glomerulus, remained constant. This fact is clearly shown in Figure 1 and Table II. Moreover, in most instances, the filtration fraction remained constant during adjustment to compression or during recovery.

B. *Tubular function during abdominal compression*

1. *Tubular reabsorption of water*

Urine flow decreased strikingly immediately after the application of abdominal compression in every subject (Table IV) even in the presence of diuresis during the control period. The reduction in filtration rate had little influence in producing this effect *per se*, since the urinary concentration usually increased markedly, indicating the operation of increased tubular water reabsorption as the cause of the oliguria.

In Table IV, the maximal concentration change is presented in terms of mannitol or inulin urine: plasma concentration ratios denoting the extent to which these substances are concentrated in the tubular urine by the active reabsorption of water.

TABLE V

Effect of increased intra-abdominal pressure upon maximal glucose reabsorptive capacity (Tmg) in normal subjects

All values except those marked with an asterisk are averages of 2 or more periods.

Subject	Sex	Age	Control				Pressure				Recovery			
			Plasma concentration glucose	Load glucose / Glucose Tm	Glucose Tm	Glomerular filtration rate / Glucose Tm	Plasma concentration glucose	Load glucose / Glucose Tm	Glucose Tm	Glomerular filtration rate / Glucose Tm	Plasma concentration glucose	Load glucose / Glucose Tm	Glucose Tm	Glomerular filtration rate / Glucose Tm
			mgm. per cent		*mgm. per min.*		*mgm. per cent*		*mgm. per min.*		*mgm. per cent*		*mgm. per min.*	
L. R.	Male	30	302	1.55	245.6	0.514	259	1.46	185.4	0.560	231	[illegible]	227.4	[illegible]
L. G.	Female	28	535	1.69	353.0	0.348	512	1.71	252.1	0.330	[illegible]*	[illegible]*	[illegible]*	[illegible]*
J. Si.	Male	17	522	2.51	327.8	0.483	516	2.42	[illegible]	[illegible]	[illegible]*	[illegible]*	[illegible]*	[illegible]*
J. Q.	Male	43	497	1.69	452.3	0.315	459	1.52	435.7	0.330	413	1.47	[illegible]	[illegible]
M. E.	Female	43	549	1.81	373.0	0.329	497	1.55	344.0	0.327	[illegible]*	[illegible]*	[illegible]*	[illegible]*

All other substances examined likewise showed an increase in concentration.

There was no evidence favoring the possibility that ureteral obstruction by the compressing abdominal cuff might account for the oliguria. Following release of the cuff, the urine flow continued to be depressed in most instances. A sudden increase of urine flow following pressure release, due to the outflow of impounded urine, was never observed. Moreover, the change in clearance values and urine flow was observed on 2 occasions when the ureters were catheterized and pressure applied to the abdominal wall.

2. *Tubular transfer maxima (Tm)*

The maximal tubular reabsorption of glucose and the maximal tubular excretion of diodrast were reduced by abdominal compression (Tables V and VI). Glucose Tm was measured in 5 subjects. In 2 (J. Q. and M. E.), the Tm value was not significantly reduced, possibly because of reduced pressure transmission. In the remaining subjects, glucose Tm fell strikingly from 35 to 24.5 per cent on the average. Greater falls during individual periods are masked by the inclusion in the average value of periods in which readjustment had taken place. Among the subjects in whom diodrast Tm was determined, significant average reductions were noted on every occasion during compression. During the recovery phase, the Tm values returned to the control level and, on several occasions, somewhat above it, possibly as a result of dead-space error. It will be noted that the reduction ranged between 39 and 23.4 per cent. In all subjects, exclusive of those in whom no significant change was observed, *the average reduction of glucose and diodrast Tm was of the same order of magnitude as the average reduction of effective renal plasma flow and glomerular filtration.*

When the relation between filtration rate and the simultaneously determined Tm value was examined, it was found that the maximal tubular transfer of either glucose or diodrast varied more or less directly with the filtration. Figures 2 and 3 illustrate this relationship. It can be seen that the ratio between Tm and filtration rate remained relatively constant throughout. Table V reveals that this ratio did not change significantly on the average in the case of maximal tubular glucose reabsorption. In 2 of the 5 subjects in whom diodrast Tm was measured, however (Table VI), there was a significant depression of the ratio (L. R. and M. P.). In these instances the inclusion of high values observed in the control period and the possible operation of intra-renal vasoconstriction may have been responsible for the alteration. On the whole, it appeared that the maximal rate of tubular transfer of glucose and diodrast was reduced by abdominal compression to about the same extent as the glomerular filtration rate and effective renal plasma flow.

Validity of the methods of the measurement. The observed reduction in maximal tubular transfer capacity could be ascribed to inadequate loading of the intracellular mechanisms by glucose or

TABLE VI

Effect of increased intra-abdominal pressure upon maximal diodrast excretory capacity (Tm_D)

All values, except those marked with an asterisk, are averages of 2 or more periods.

Subject	Sex	Age	Control: Plasma concentration diodrast	Control: Load diodrast / Diodrast Tm	Control: Diodrast Tm	Control: Glomerular filtration rate / Diodrast Tm	Pressure: Plasma concentration diodrast	Pressure: Load diodrast / Diodrast Tm	Pressure: Diodrast Tm	Pressure: Glomerular filtration rate / Diodrast Tm	Recovery: Plasma concentration diodrast	Recovery: Load diodrast / Diodrast Tm	Recovery: Diodrast Tm	Recovery: Glomerular filtration rate / Diodrast Tm
			mgm. per cent		*mgm. per min.*		*mgm. per cent*		*mgm. per min.*		*mgm. per cent*		*mgm. per min.*	
M. S.	Female	50	12.8	1.07	50.1	1.94	11.6	1.05	36.9	2.09	11.4	1.13	47.3	2.08
L. R.	Male	30	14.5	1.37	44.5	2.23	13.1	0.95	36.4	1.75	—	—	—	—
M. P.	Male	16	16.0	1.92	40.5	2.84	17.7	2.11	26.6	2.12	18.5*	2.06*	48.6*	2.03*
L. G.	Female	28	19.0	2.60	41.5	2.67	17.8	2.37	31.8	2.59	16.3*	2.45*	47.6*	2.53*
J. W.	Female	29	23.0	2.96	57.2	2.17	17.9	2.24	34.8	2.33	14.2	2.14	55.4	2.61

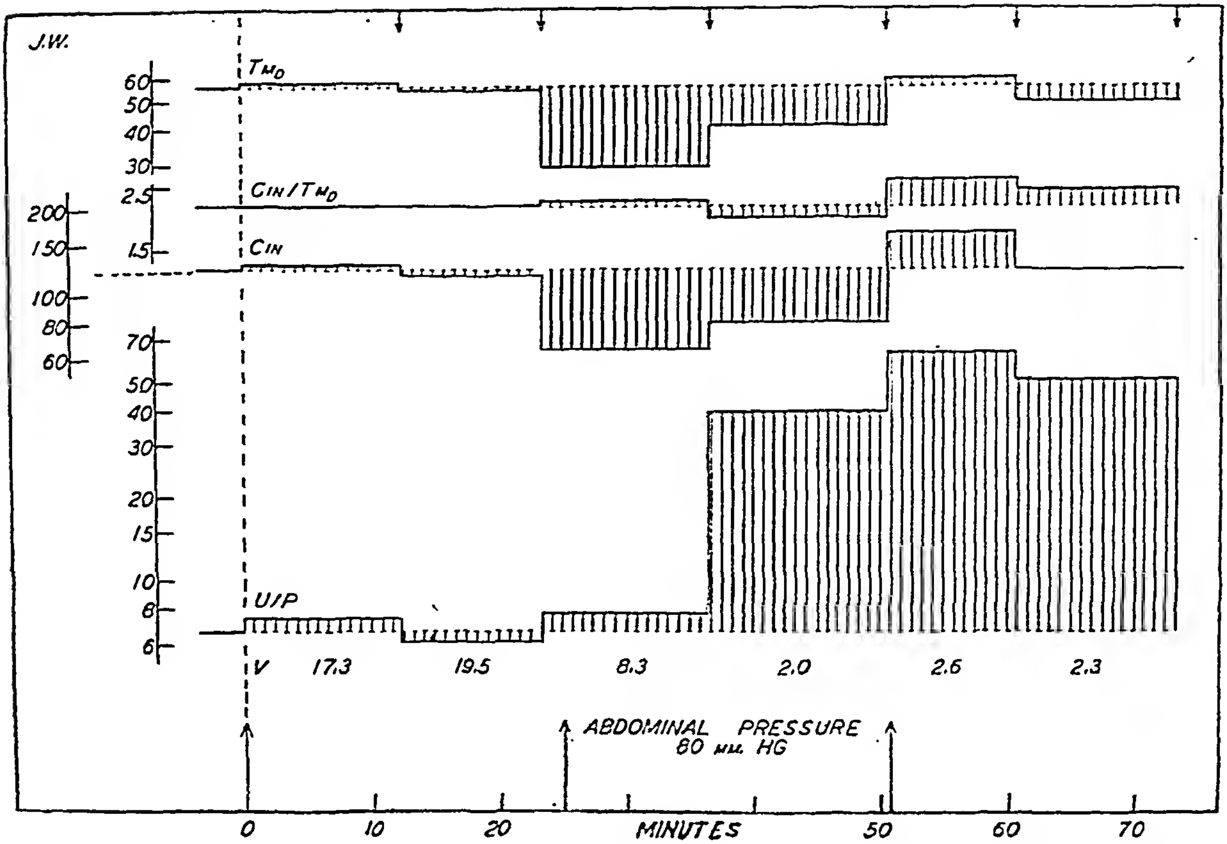

FIG. 2. ALTERATION IN MAXIMAL TUBULAR DIODRAST EXCRETORY RATE (DIODRAST TM) AND GLOMERULAR FILTRATION RATE DURING ABDOMINAL COMPRESSION IN SUBJECT J. W.

Diodrast Tm (Tm_D) (mgm. per min.) and glomerular filtration rate (C_{IN}) (ml. per min.) fell during increased intra-abdominal pressure while the inulin U/P ratio rose. The relation between glomerular filtration rate and diodrast Tm (C_{IN}/Tm_D) remained unchanged throughout. It can be seen that both diodrast Tm and the filtration rate increased late in the period of pressure application. Urine flow (V) (ml. per min.) was markedly depressed.

diodrast, either as a result of over-all renal ischemia and lowered filtration rate or focal ischemia and localized slowing of filtration. In the first instance, the smaller carriage of diodrast or glucose would result in a failure to supply sufficient material for saturation of the whole kidney and, in the second, adequate over-all loading would mask focal ischemia and failure to saturate tubular transfer mechanisms in discrete localized regions.

a. over-all loading. The calculated load of glucose presented to the tubules for reabsorption should exceed the tubular capacity by 20 per cent and the quantity of diodrast carried in the plasma to the tubules [3] should exceed the excretory capacity by 100 per cent (8) to assure adequate saturation. In every instance the load to Tm ratio for glucose was greater than 1.2 (Table V). On 2 occasions (M. S. and L. R.), the diodrast load fell below the prescribed limit (Table VI). Considering the fact that the decrements in diodrast Tm in

[3] The load of glucose brought in the glomerular filtrate to the tubule cells for reabsorption was calculated as the arterial plasma concentration of glucose (mgm. per ml.) times the filtration rate (ml. per min.). The diodrast load is the amount of diodrast carried in the plasma to the tubules less that removed by filtration. It was calculated as the plasma concentration of diodrast (mgm. per ml.) times the estimated effective renal plasma flow, less the plasma concentration of diodrast (mgm. per ml.) multiplied by the filtration rate (ml. per min.) and a factor (0.72) to correct for protein binding. Since effective renal plasma flow cannot be determined at high plasma levels of diodrast, it was estimated on the basis of the observed filtration rate, assuming a normal value of 20 per cent for the filtration fraction.

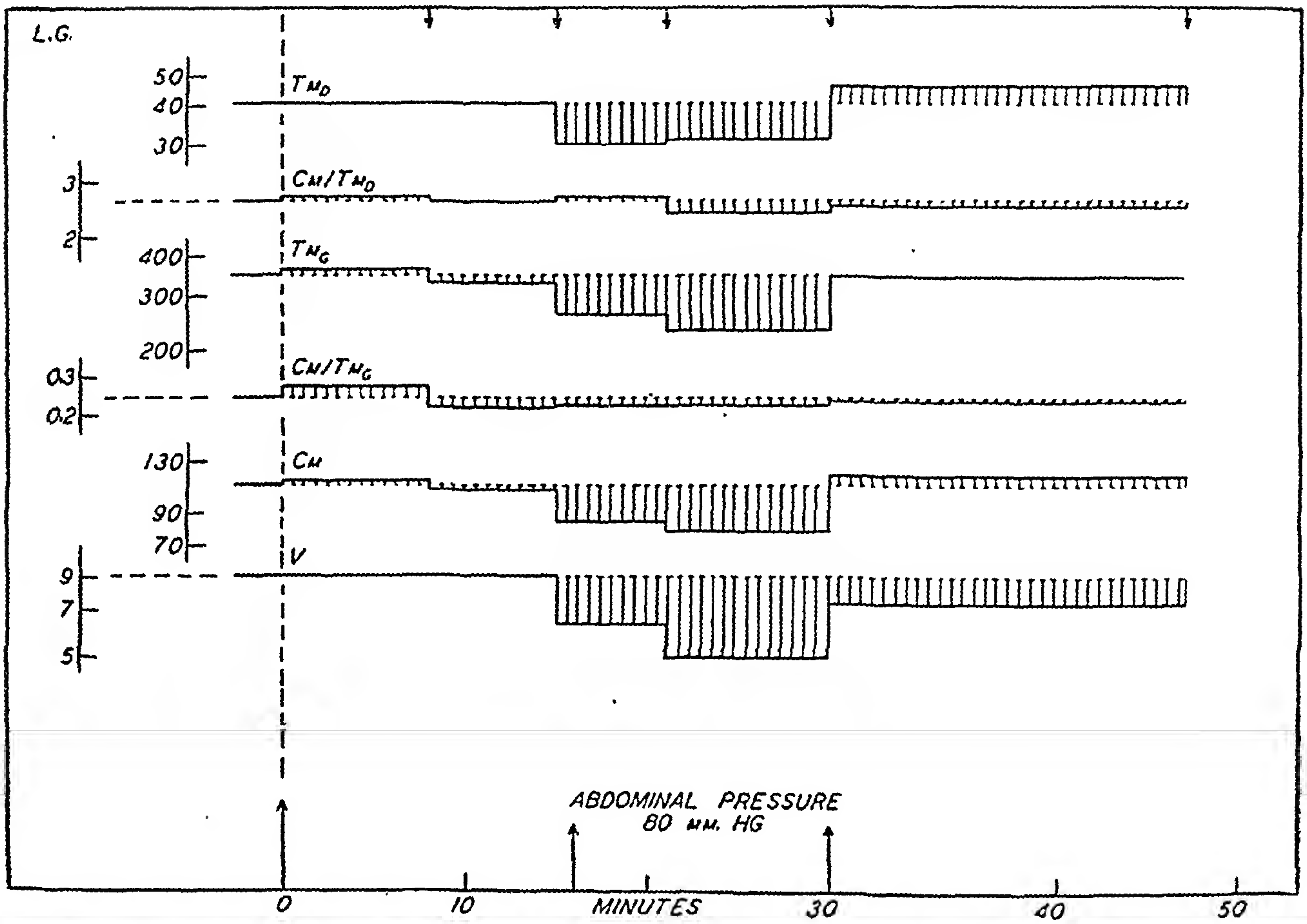

FIG. 3. ALTERATIONS OF MAXIMAL TUBULAR DIODRAST EXCRETORY RATE (DIODRAST TM) AND MAXIMAL TUBULAR GLUCOSE REABSORPTION RATE (GLUCOSE TM) DURING ABDOMINAL COMPRESSION IN SUBJECT L. G.

Diodrast Tm (Tm_D) (mgm. per min.) and glucose Tm (Tm_G) (mgm. per min.) fell equally during compression. *The relationships between the Tm values and the glomerular filtration rate measured by the mannitol clearance* (C_M) remained constant. Urine flow (V) decreased, but returned toward the control level following the termination of the period of pressure.

these cases during pressure were of the same magnitude as those where the calculated loads were sufficiently high, it is possible that the calculated loads were erroneous. The calculation of diodrast load requires an estimation of the renal plasma flow on the basis of the filtration rate, assuming a normal filtration fraction of 20 per cent. It is not unlikely that this assumption was in error in these instances and that the actual plasma flow was much greater. In any case, it is evident that inadequate loading cannot be the cause of the reduction in tubular activity during increased intra-abdominal pressure in most of these studies.

b. focal loading. Despite the demonstration that adequate loading did not prevent the fall in Tm observed during pressure, it might be argued that these figures, referable to the kidney as a whole, may fail to reveal focal inadequacies. This argument may be visualized and examined as follows.

For the purposes of discussion, the kidney may be divided into 4 concentric zones (Table VII)

TABLE VII

Zone	Reduction	Filtration rate	Plasma flow	Load glucose	Reabsorbed glucose	Load diodrast	Excreted diodrast
		ml. per min.	*ml. per min.*	*mgm. per min.*	*mgm. per min.*	*mgm. per min.*	*mgm. per min.*
I	80%	6	25.0	36	36	6.3	6.3
II	50%	15	62.5	90	90	15.5	13.0
III	10%	27	112.5	162	100	27.8	13.0
IV	0%	30	125.0	180	100	31.0	13.0
Over-all effect	35%	78	325.0	468	326	80.6	45.3
Total reduction	35%	35%	35%		18.4%		12.9%

throughout each of which blood flow and filtration are regularly distributed, although great differences may exist between zones.[6] It may be further assumed that the effect of compression is different in each zone (as the result of a gradient of pressure, for example, so that the compressional force is greater at the periphery than at the center), but that the effect in each zone is constant and regular throughout the entire region. During the control period, prior to compression, the over-all glomerular filtration rate, blood flow and transfer maxima of glucose and diodrast are equally divided among all the zones. The over-all glomerular filtration rate may be taken as 120 ml. per minute, the renal plasma flow as 500 ml. per minute, glucose Tm, 400 mgm. per minute, and diodrast Tm, 52 mgm. per minute, while the glucose and diodrast plasma levels are maintained at 600 mgm. per cent and 30 mgm. per cent, respectively. With the application of a compressional force, the plasma flow and filtration rate may be assumed to undergo reductions of 80, 50, 10, and 0 per cent in zones I to IV, respectively. The data tabulated in Table VII reveal that the percentile reductions in over-all Tm thus produced do not equal the percentile reductions in plasma flow and filtration rate, and the reductions in diodrast and glucose Tm are not equivalent. The discrepancy in Tm values could be expected to vary greatly, depending upon the relative loading of the 2 tubular mechanisms. Therefore, it appears that the changes in tubular function observed during abdominal compression cannot be ascribed to focal ischemia due either to focal vasoconstrictive activity or to the operation of a gradient of pressure across the kidney.

DISCUSSION

It has been found that abdominal compression increases the pressure equally throughout the abdominal cavity and in doing so produces striking renal functional changes. Renal plasma flow and glomerular filtration are almost always reduced, together with the tubular activities of maximal glucose reabsorption and maximal diodrast excretion. All these functions are reduced to the same degree. Water reabsorption, on the other hand, is always enhanced, the urine flow decreases, and the urine becomes more concentrated.

Every effort was made to eliminate experimental error. Determinations of the renal extraction of sodium p-aminohippurate have validated the use of the clearance technique in measuring the changes of renal blood flow. Arteriovenous shunting has been definitely ruled out by this method. Likewise, the alterations in glucose and diodrast Tm may be accepted as indicative of changes in tubular function, since it appears that inadequate loading or focal ischemia may be excluded as possible misleading factors.

On the basis of the averaged data, it seems likely that the increase of venous pressure during abdominal compression is usually sufficient to account for the reduction of renal plasma flow. Excluding J. S. and J. Q. (Table II) from consideration, the averaged figures may be used in calculating the reduction to be expected on the basis of an increment of venous pressure alone.[7] If the pressure gradient across the renal circuit is assumed to change from 85 mm. Hg to 70 mm. Hg during compression as a result of an increase of renal venous pressure from 5 mm. Hg to 20 mm. Hg (the mean renal arterial pressure remaining constant at 90 mm. Hg), and, if the renal resistance does not change, the average renal plasma flow of 621 ml. per minute would fall to 512 ml. per minute. Actually, it was observed to fall to 488 ml. per minute, a somewhat greater decrease, which would require a venous pressure increment of 18 mm. Hg. It can be seen that the elevation of venous pressure alone is approximately sufficient to account for the observed change.

The reduction of renal plasma flow was much greater than average immediately following the application of pressure in many subjects and

[6] It is obvious that the kidney may be further subdivided until each zone refers to a single nephron. Hence, what is said here with reference to 4 arbitrary and regularly placed renal zones would apply with equal force to discrete units distributed irregularly through the kidney.

[7] In any vascular bed of constant resistance, the relation of blood or plasma flow to the loss of pressure from the beginning to the end of the circuit remains constant. This relationship may be expressed as follows, where F is the initial renal plasma flow; P, the initial difference in renal arterial and renal venous pressures; F' and P', these values when the pressure difference has been altered as with an increment of venous pressure

$$F'/F = P'/P \text{ and}$$
$$F' = P'F/P$$

throughout the entire study in J. S. Although these facts may indicate the operation of vasoconstriction, it is not at all unlikely that venous pressure, in these instances, was higher than average as a result of compressional contraction of the abdominal musculature. The intra-abdominal pressure may be raised as high as the arterial pressure by this maneuver. On the other hand, muscular contraction may lower intra-abdominal pressure by thrusting away the pressure girdle, and it is possible that the adjustment to compression noted in some instances, where renal function gradually improved, may have arisen from this factor rather than from the release of vasoconstriction.

Although the increase in renal venous pressure seems to provide an adequate cause for the reduction in renal blood flow, it is at first difficult to account for the other changes in renal function on this basis. Certainly one might expect a change in filtration fraction similar to that attributed to efferent arteriolar vasoconstriction (18), since the elevation of the pressure gradient in the renal vascular bed should increase effective filtration pressure and the filtration rate should remain relatively constant despite the reduction in blood flow. Since an increment of venous pressure is equally effective in all parts of the renal vascular bed, there is no reason to expect a fall in diodrast and glucose Tm. The fall in these values indicates the cessation of function in a number of nephrons and glomeruli proportional to the decrement in Tm (19).

The closure of afferent arterioles would produce changes in renal function similar to those observed. In obstructing blood flow, arteriolar closure would halt filtration in the glomerulus and, in consequence, abolish glucose reabsorption and diodrast excretion in the nephron dependent upon it. Although the tubule might remain in a normal state, it is obvious that glucose would no longer be brought to it for removal and that excreted diodrast would not be washed into the renal pelvis. Hence, the nephron would cease to contribute to Tm and the values for it would be reduced. In the remainder of the kidney, renal blood flow and glomerular filtration rate must continue undisturbed, since the filtration fraction and the relation between the clearance values and Tm are not altered by compression. Thus, afferent arterioles would be required to close completely or remain unconstricted. While this hypothesis satisfactorily accounts for many of the changes in renal function, it fails to consider the implications of the elevation of venous pressure.

It has been pointed out above that the increase in venous pressure is probably sufficient to account for the observed reduction in renal blood flow, but, under the conditions of afferent arteriolar constriction, it would be necessary to assume that increased resistance to flow is entirely responsible and that the venous pressure increment is without effect. It must be admitted, however, that diminished resistance in the remainder of the renal vascular bed is necessary to offset the rise in venous pressure. If such a vasodilation occurred in the efferent arterioles it is possible that blood flow and filtration rate might maintain a constant relationship to one another and to the Tm values as observed. However, it seems extremely unlikely that both vasoconstriction and vasodilation occur simultaneously in the kidney. An adjustment of efferent arteriolar tone in response to renal venous pressure has not been observed in other conditions, and it seems highly improbable that it occurs during abdominal compression. For these reasons the hypothesis that afferent arteriolar vasoconstriction results in functional amputation of a portion of the renal parenchyma may be dismissed as unacceptable.

In the discussion thus far, the fact that the pressure rises in the renal pelvis, opposing the outflow of urine, has not been considered. If the resistance to the flow of urine differs in different nephrons (as it may, since the tubules vary considerably in length), there may be a wide range of pressures at the terminal ends of the tubules. Elevation of pelvic pressure would then block urine outflow from those tubules in which the terminal pressures are lower than pelvic pressure. As a result, Tm values and the glomerular filtration rate would fall. In this situation the blood perfusing inactive glomeruli would continue to be cleared of diodrast or p-aminohippurate by neighboring operative tubules and the filtration fraction would *fall* as a result of the reduction of filtration rate independently of blood flow. Since the renal venous pressure rises during abdominal compression, however, the filtration fraction in the active glomeruli would be *increased* and might be expected to mask the

effect of the clearance of diodrast or p-aminohippurate from blood perfusing inactive glomeruli.

This hypothesis has the advantage of providing a simple explanation for all the changes observed in this study. Vasoconstriction would be unnecessary, the fall in blood flow being entirely accounted for by the increment in renal venous pressure. The demonstration by others (18) that the filtration rate in man tends to remain unchanged is supported by the finding that it does not change in the residual active glomeruli. Such reduction of filtration rate and Tm values as occurs is entirely attributable to the blockage of flow from "low-pressure" nephrons by the elevated pelvic pressure. It should be noted that equal elevations in renal venous pressure and pelvic pressure produce equal percentile changes in renal blood flow and in the mass of functioning tubular tissue, respectively, according to this hypothesis. The implications of such a relationship are not clear and are under further investigation.

Increased intrapelvic and intratubular pressure may be instrumental in raising the urinary concentration by stimulating the mechanisms of water transfer to greater activity. However, the psychic and physical stimulus of compression should provoke the release of antidiuretic hormone by the pituitary, since less severe stimuli have been found to have this action (20). In 1 subject with documented diabetes insipidus presenting marked diuresis as a result of withdrawal of pitressin, abdominal compression caused oliguria.

The pathological renal physiology of increased intra-abdominal pressure has been clarified by this study. Under the conditions imposed, retention of water and probably of salt occurs in spite of the presence of factors conducive to diuresis. The reduction of glomerular filtration rate and oliguria, if maintained for any length of time, would certainly prevent the normal excretion of urea, and azotemia would develop.

For the short period of time during which positive G is experienced by airmen, abdominal compression sustains the cardiac output by the expression of blood into the thorax from the abdomen and by minimizing the gravitational effects upon the flow of blood from the inferior venous chamber into the superior chamber. The simultaneous reduction of arterial blood flow into the splanchnic bed also results in the diversion of a greater proportion of the cardiac output to the brain. It is likely that prolonged application of increased intra-abdominal pressure may be detrimental because the cardiac output is ultimately reduced by about the same extent as the blood flow through the kidneys and liver (21, 22). Hypotension and faintness were observed on several occasions during the course of this study in subjects not reported herein.

SUMMARY

The effective renal plasma flow, glomerular filtration rate, and tubular function have been measured in 17 normal human subjects by the clearance and saturation techniques during the application of a pneumatic abdominal girdle under pressure. Measurement of the venous pressure in the inferior vena cava and renal vein following venous catheterization revealed that the compressional pressure of 80 mm. Hg used in this study raised intra-abdominal venous pressure to about 20 mm. Hg.

The effective renal plasma flow and glomerular filtration rate were always reduced by increased intra-abdominal pressure, on the average of 24.4 per cent and 27.5 per cent, respectively. The filtration fraction, or the percentage of the plasma flow filtered at the glomerulus, showed no significant change.

It was found that the elevation of renal venous pressure was probably sufficient to account for the reduction of renal plasma flow.

The extraction of sodium p-aminohippurate by the kidney was not altered, indicating the validity of the clearance method for measuring renal plasma flow under these conditions and demonstrating the absence of arteriovenous shunting.

Maximal tubular diodrast excretion (diodrast Tm) was reduced significantly in all 5 subjects, while maximal tubular glucose reabsorption (glucose Tm) fell significantly in 3. The percentile decrease in both values was equal to that in effective renal plasma flow and glomerular filtration rate. It appears that this phenomenon cannot be attributed to inadequate loading of tubular transfer mechanisms, as a result of either diffuse or focal ischemia.

The theoretical implications of these findings are discussed.

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BIBLIOGRAPHY

1. Thorington, J. M., and Schmidt, C. F., A study of urinary output and blood-pressure changes resulting in experimental ascites. Am. J. M. Sc., 1923, 165, 880.
2. Swick, M., Intravenous urography by means of the sodium salt of 5-iodo-2-pyridon-N-acetic acid. J. A. M. A., 1930, 95, 1403.
3. Jaches, L., and Swick, M., Opaque media in urology, with special reference to a new compound, sodium ortho-iodohippurate. Radiology, 1934, 23, 216.
4. Carlson, H. E., The proven ineffectiveness of the compression bag in intravenous pyelography. J. Urol., 1946, 56, 609.
5. Wood, E. H., Lambert, E. H., Code, C. F., and Baldes, E. J., Factors involved in the protection afforded by pneumatic anti-blackout suits. N.R.C., C.M.R., C.A.M. Report No. 351, August 24, 1944.
6. Wilkins, R. W., Friedland, C. K., and Bradley, S. E., Estimations of cardiac and vasomotor reserve, especially in response to strains designed to simulate those of acceleration. N.R.C., C.M.R., C.A.M. Report No. 177, September 1, 1943.
7. Goldring, W., Chasis, H., Ranges, H. A., and Smith, H. W., Relations of effective renal blood flow and glomerular filtration to tubular excretory mass in normal man. J. Clin. Invest., 1940, 19, 739.
8. Goldring, W., and Chasis, H., Hypertension and hypertensive disease. The Commonwealth Fund, New York City, 1944.
9. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. J. Clin. Invest., 1945, 24, 388.
10. Fujita, A., and Iwatake, D., Bestimmung des echten Blutzuckers ohne Hefe. Biochem. Ztschr., 1931, 242, 43.
11. Nelson, N., A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 1944, 153, 375.
12. Cournand, A., and Ranges, H. A., Catheterization of the right auricle in man. Proc. Soc. Exper. Biol. & Med., 1941, 46, 462.
13. Moritz, F., and von Tabora, D., Über eine Methode, beim Menschen den Druck in oberflächlichen Venen exakt zu bestimmen. Deutsch. Arch. f. Klin. Med., 1910, 98, 475.
14. Wilkins, R. W., and Bradley, S. E., Changes in arterial and venous blood pressure and flow distal to a cuff inflated on the human arm. Am. J. Physiol., 1946, 147, 260.
15. Smith, H. W., Physiology of the renal circulation. Harvey Lectures, 1939–1940, Ser. 35, 166.
16. Bradley, S. E., and Bing, R. J., Renal function in the harbor seal (Phoca vitulina L.) during asphyxial ischemia and pyrogenic hyperemia. J. Cell. and Comp. Physiol., 1942, 19, 229.
17. Senator, H., Albuminuria in health and disease. The New Sydenham Society, Selected Monographs, 1884, 110, 1.
18. Smith, H. W., Chasis, H., Goldring, W., and Ranges, H. A., Glomerular dynamics in the normal human kidney. J. Clin. Invest., 1940, 19, 751.
19. Smith, H. W., Lectures on the kidney. Porter Lectures—Series IX. University Extension Division, University of Kansas, Lawrence, Kansas, 1943.
20. Theobald, G. W., The repetition of certain experiments on which Molitor and Pick base their water-centre hypothesis, and the effect of afferent nerve stimuli on water diuresis. J. Physiol., 1934, 81, 243.
21. Wilkins, R. W., Halperin, M., and Bradley, S. E., Unpublished data.
22. Bradley, S. E., Studies of hepatic blood flow in man. J. Clin. Invest., 1946, 25, 918.

THE DIALYZABILITY OF THE PRESSOR AND ANTIDIURETIC ACTIVITIES OF PITRESSIN

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According to the present extent of our knowledge it appears that the pressor and the antidiuretic activities of Pitressin[1] both reside in the same substance. Since Kamm (1) showed that the pressor activity dialyzes from aqueous solution, the antidiuretic activity should dialyze also. But Ralli (2) found that when Pitressin mixed with normal urine was dialyzed against water the full antidiuretic activity for rats remained in the non-dialyzable fraction. This suggested that urine might in some way inhibit the dialysis of Pitressin. On the other hand, if pressor activity dialyzes and antidiuretic does not, dialysis would provide a means of separating the 2 activities.

Others (3) have reported that antidiuretic activity does not dialyze. However, Smith (4) early showed that all 3 posterior pituitary activities dialyzed, and Walker (5) demonstrated the presence of antidiuretic activity in the dialysate.

The purpose of the present experiment is to report the pressor activity for dogs and the antidiuretic activity for humans of the non-dialyzable residue following dialysis of Pitressin mixed with water and mixed with normal urine. The results suggest that both the pressor and the antidiuretic activities do dialyze, whether mixed with water or with urine.

PREPARATION OF SOLUTIONS

Five tests were made on each of 4 human subjects using the following preparations:

A. No injection—control
B. Pitressin (not dialyzed)
C. Pitressin dialyzed from water
D. Pitressin dialyzed from urine I
E. Pitressin dialyzed from urine II

Solution C. Twenty-five ml. of Pitressin containing 20 p.u. per ml. (total 500; p.u. = pressor units) was dialyzed for 6 hours in 24/32 inch Visking tubing against running distilled water at room temperature and with agitation of the tubing. The dialysis residue was diluted to 100 ml. with water containing 0.5 per cent chloretone. The solution (representing 5 p.u. per ml.) was filtered through a Seitz filter using a 1-inch E.K. pad.

Solution B. With no dialysis, 25 ml. of Pitressin was diluted in the same way and subjected to the same Seitz filtration. This filtration of the control would make it possible to detect any adsorption of pressor or antidiuretic activity by the filter pad.

Solution D. Ten ml. (200 p.u.) of Pitressin was mixed with 26 ml. of diuretic urine from a normal human being. This urine contained 2.41 mgm. of solids per milliliter. The mixture was dialyzed as above; the dialysis residue was made to 40 ml. with water and Seitz filtered. The final solution represented 5 p.u. per ml.

Solution E. To eliminate the possibility that the urine effect described by Ralli was quantitatively dependent on some urinary constituent, it seemed desirable to increase the ratio of urinary solids to pressor units. Ten ml. (200 p.u.) of Pitressin was mixed with 1,000 ml. of normal human urine having pH 5.82, solids 36.6 mgm. per ml. This solution was dialyzed overnight (17 hours) to specific resistance 14,100 ohms and pH 6.05. One-fourth of the dialysis residue was dried by appropriate means, and the resulting powder was taken up in 10 ml. of sterile water containing chloretone. The preparation was an amber liquid containing suspended material. Each milliliter represented 5 p.u. and 25 ml. of the normal urine.

PROCEDURE

The antidiuretic activity was tested in 4 normal human subjects who continued their usual work during the test period. Each solution was tested in all 4 subjects on the same day. No food or water was taken after 10 p.m. of the preceding night, the bladder was emptied at 8 a.m., the test solution was injected, and 1,000 ml. of water was taken by mouth at the same time. The dose in each case was 0.1 ml. representing 0.5 p.u., which was given into the muscle of the upper arm. Urine was voided at 30-minute intervals and the cumulative excretion plotted against time as shown in Figure 1. Each solution was also assayed for pressor activity in dogs.

RESULTS

In every subject urine excretion was significantly delayed by 0.5 p.u. of undialyzed Pitressin, as shown in Figure 1. On the other hand, none of the dialyzed samples, whether from water or urine, resulted in a comparable delaying effect; the effect

[1] Pitressin is a registered trade mark of Parke, Davis and Company.

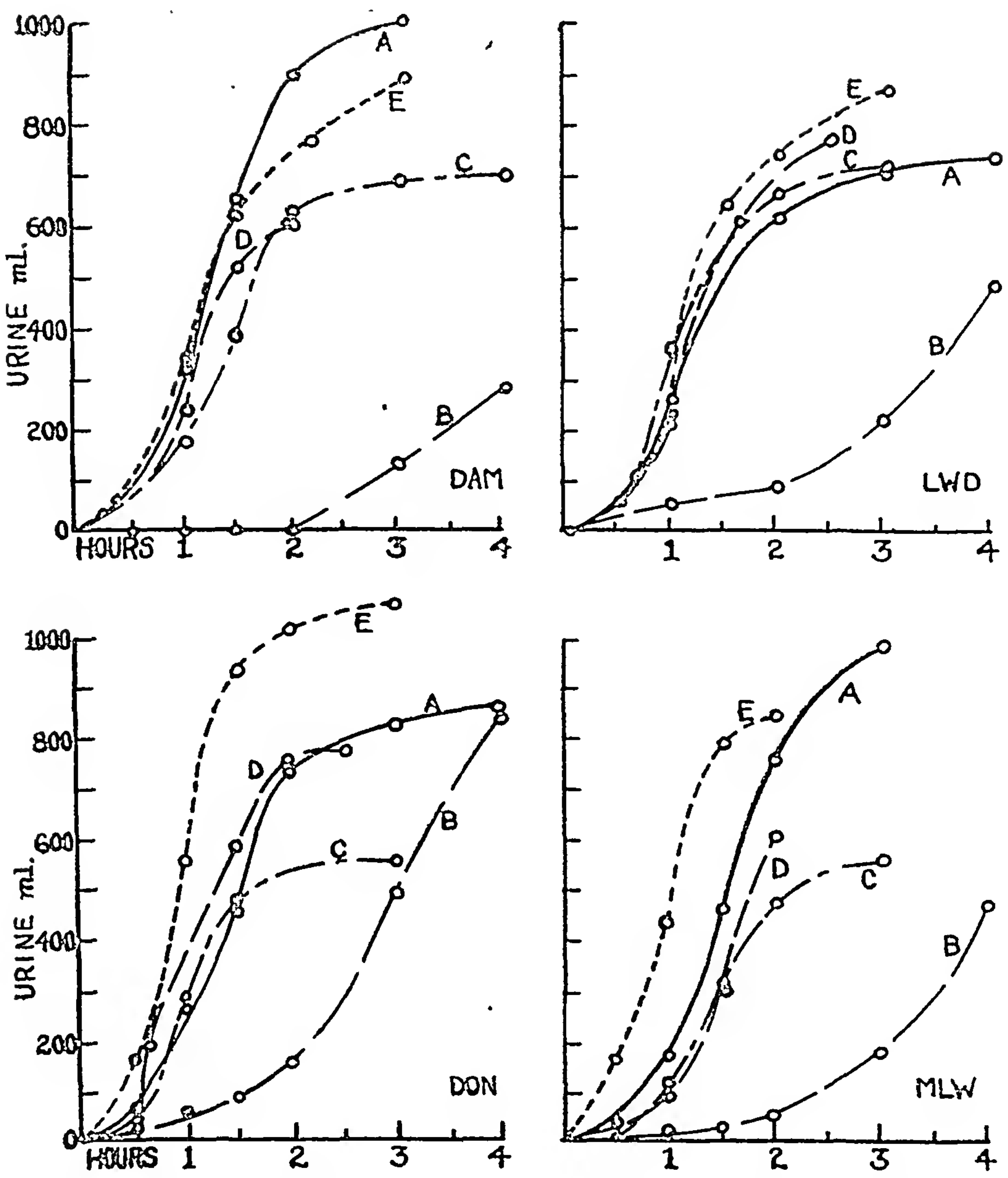

Fig. 1. Effect of Dialysis on Antidiuretic Activity of Pitressin Solutions

A. No injection—control.
B. Pitressin not dialyzed.
C. Pitressin dialyzed from water.
D. Pitressin dialyzed from urine I.
E. Pitressin dialyzed from urine II.

of such samples was more nearly like that of "no injection."

The pressor assay report was as follows:

B. Pitressin. Not dialyzed— 4.5 p.u. per ml.
C. Pitressin dialyzed from water— Much less than 0.1 p.u. per ml.
D. Pitressin dialyzed from urine I— Less than 0.1 p.u. per ml.
E. Pitressin dialyzed from urine II— Less than 0.1 p.u. per ml.

Thus, the pressor activity remained in the undialyzed preparation, but was lost from all dialyzed solutions.

CONCLUSIONS

When mixtures of Pitressin with water or with urine were dialyzed, both the pressor and antidiuretic activities disappeared from the dialysis residue.

The author wishes to express appreciation to Dr. D. A. McGinty for helpful suggestions, to Mr. L. W. Rowe for pressor assays, to Miss M. L. Wilson, Mr. D. C. Neubaum and Miss S. C. McKinsey for technical assistance.

BIBLIOGRAPHY

1. Kamm, O., The dialysis of pituitary extracts. Science, 1928, 67, 199.
2. Ralli, E. P., Robson, J. S., Clarke, D., and Hoagland, C. L., Factors influencing ascites in patients with cirrhosis of the liver. J. Clin. Invest., 1945, 24, 316.
3. Gilman, A., and Goodman, L., The secretory response of the posterior pituitary to the need for water conservation. J. Physiol., 1937, 90, 113.
   Boylston, G. A., and Ivy, A. C., An antidiuretic substance present in the urine of dehydrated rats. Proc. Soc. Exper. Biol. & Med., 1938, 38, 644.
   Ingram, W. R., Ladd, L., and Benbow, J. T., The excretion of antidiuretic substance and its relation to the hypothalamico-hypophyseal system in cats. Am. J. Physiol., 1939, 127, 544.
   Teel, H. M., and Reid, D. E., Observations upon the occurrence of an antidiuretic substance in the urine of patients with pre-eclampsia and eclampsia. Endocrinology, 1939, 24, 297.
   Robinson, F. H., and Farr, L. E., The relation between clinical edema and the excretion of an antidiuretic substance in the urine. Ann. Int. Med., 1940, 14, 42.
4. Smith, M. I., and McClosky, W. T., On the dialysis of the physiologically active constituents of the infundibulum. J. Pharmacol. & Exper. Therap., 1924, 24, 391.
5. Walker, A. M., Experiments upon the relation between the pituitary gland and water diuresis. Am. J. Physiol., 1939, 127, 519.

PROTEINS IN THE COLLOIDAL GOLD REACTION

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The colloidal gold test developed almost 35 years ago by Lange (1) has been utilized in studies of cerebrospinal pathology and of liver dysfunction with little more than a general understanding of the basic reasons for the production of the several reaction patterns. Rationalization of the mode of reaction by experimenting with blood protein fractions obtained by the usual salting out methods is difficult, if not impossible, because the fractions so obtained are mixtures (2) of proteins defined arbitrarily.

A promising line of attack upon the problem has been opened up by newer methods for the separation of the blood proteins (3, 4, 5), particularly those separable as electrophoretically homogeneous fractions. It has already been established (6, 7) that the gamma globulin fraction is responsible for the coagulation of the gold sol. The protective action postulated for the serum albumin, however, never has been demonstrated conclusively with concentrations likely to be encountered in body fluids. In investigating the use of the colloidal gold reaction for the detection of liver damage, the authors have used blood protein fractions prepared by the newer methods in an attempt to determine the relationships between relative concentrations of the protein fractions and the reaction curves obtained.

MATERIALS

The blood protein fractions[1] used in this study were described as follows:

Fraction	Plasma preparation no.	Distribution of electrophoretic components						
		γ	F	β_2	β_1	α_2	α_1	Alb.
II–1,2	184	96		4				
V	185					1	2	97
III–0 total*	175		18	13	64	5		
IV–1*	185			9		18	40	33‡
IV–3,4†	184			2	35	26	21	16

* Rich in lipoprotein.
† Poor in lipoprotein.
‡ Figures for albumin and α_1 are approximate due to difficulty in resolving fraction.

METHODS

The methods of preparing the gold sol and performing the tests were those recently described by Lange (8, 9, 10). Thus, the tests were performed with comparable gold sols on dilutions of experimental material in phosphate buffer, pH 7.4, and the results were read by comparison with prepared standards of graded color values.

In all cases, the plasma protein fractions were prepared in desired concentrations by dissolving them in the phosphate buffer. In later phases of the investigation, concentrations were based upon protein content as calculated from Kjeldahl determinations ($N \times 6.25$).[2]

In performing the tests, serial dilutions of each solution under test, 0.5 ml. per tube, were prepared in such manner that the dilutions increased by one-third with each successive tube. To each of these, 2.5 ml. of colloidal gold sol were added and the tubes were shaken and allowed to stand at room temperature for 2 hours. Readings were then made against a graded series of standards varying from 0 (unchanged) to 20 (colorless) after the manner suggested by Lange (8).

RESULTS

Figure 1 shows graphically the differing effects of equivalent concentrations of the several protein fractions upon the gold sol. These graphs have been plotted for each fraction from results obtained on 20 serial dilutions prepared after the manner described above. The horizontal axes of these

[1] The products of plasma fractionation employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. The authors wish to express their gratitude to Dr. John T. Edsall of Harvard University who furnished these materials.

[2] The use of the conventional nitrogen factor for fractions containing alpha and beta globulins with lipids results in some inaccuracy.

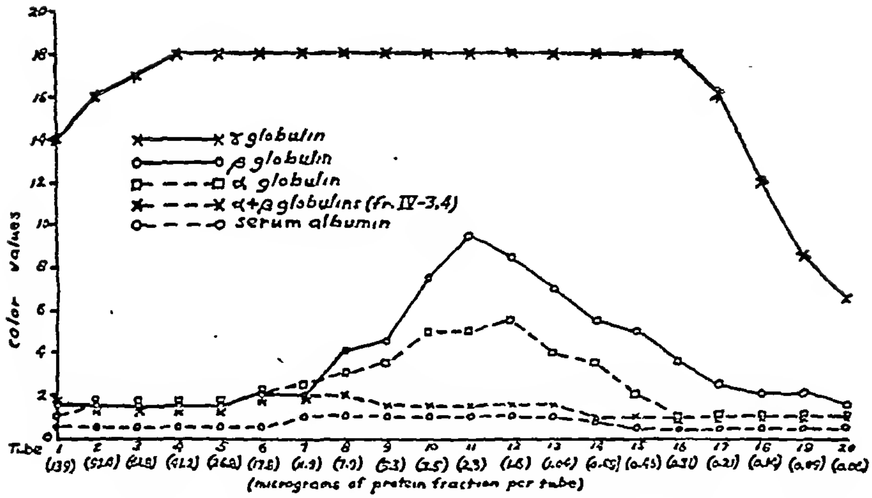

FIG. 1. EFFECT OF PROTEIN FRACTIONS ON 2.5 ML. GOLD SOL (pH 7.4)

graphs and of those to follow show uniform spacings between successive dilutions; this gives the same effect as a semi-logarithmic scale with the abscissa showing the logarithms of increasing dilutions. It will be noted that neither serum albumin nor fraction IV-3,4 containing a mixture of alpha and beta globulins exerted any significant coagulatory effect upon the gold sol whereas the gamma globulin was the only fraction with unequivocally coagulatory effects. Fractions III-0 and IV-1, one containing alpha globulin and the other beta globulin, showed such slight reactivity in the range of concentrations between 1 and 5 μg. per tube that it seems unlikely that this resulted from any specific coagulatory effect exerted by these globulins *per se*. Such minimal activity is more likely to have resulted from the presence of other substances in these mixtures. Likewise, the possible role of impurities in the gamma globulin fraction acting as protective colloids cannot be ignored entirely in the interpretation of the apparent slight protection of the sol by concentrations of that fraction above 60 μg. per tube.

Figure 2 shows the apparent effect of serum albumin upon the coagulatory action of the gamma globulin fraction. Critical analysis of the graphs shows that the protective action of this fraction is nil[3] since the critical point at which the coagula-

[3] Since these experiments were completed Lange has published data obtained with the use of horse serum al-

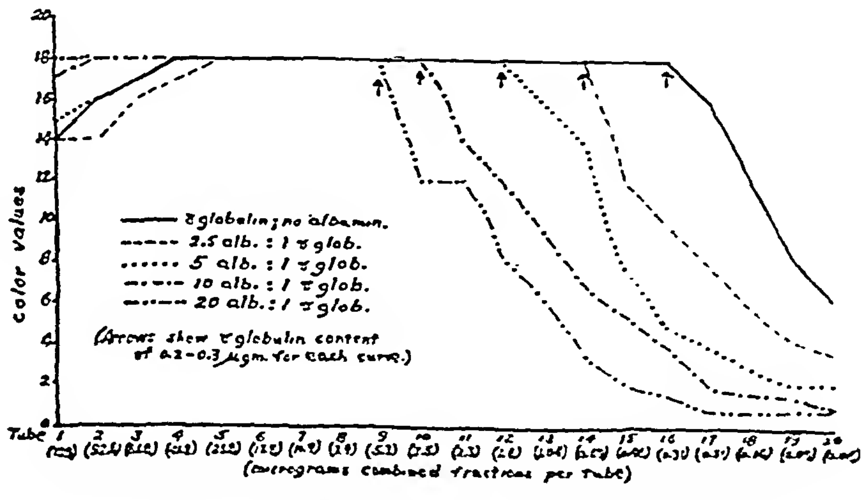

FIG. 2. EFFECT OF SERUM ALBUMIN ON γ GLOBULIN CURVE (pH 7.4)

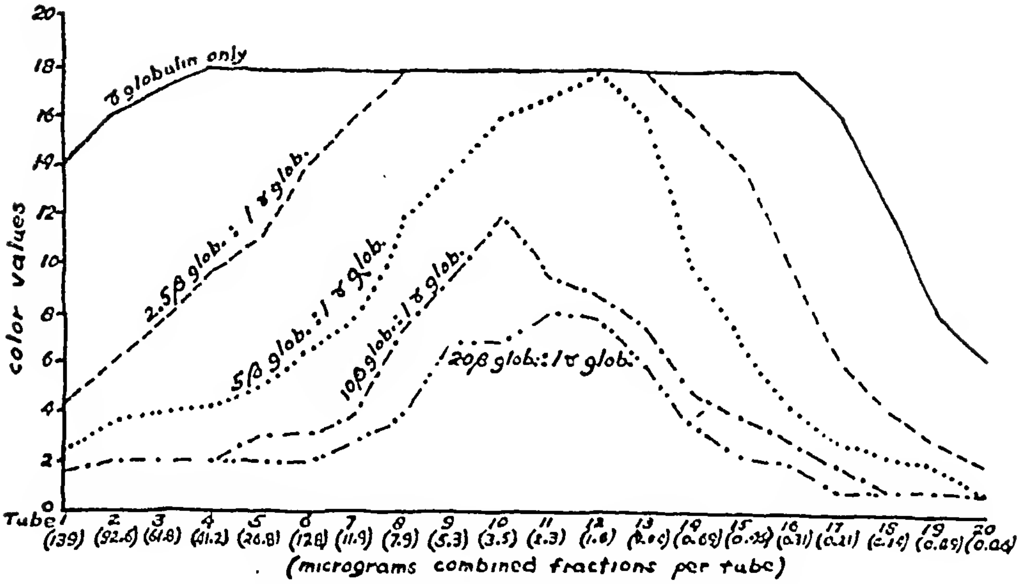

FIG. 3. EFFECT OF β GLOBULIN (FR. III-0) ON γ GLOBULIN CURVE (pH 7.4)

tory reaction diminishes sharply is determined by the actual amount of gamma globulin present in the tube and not upon the albumin to globulin ratio. This critical point is reached when the concentration of gamma globulin drops below 0.3 μg. per 2.5 ml. of gold sol as shown by notations on the chart.

The effect of the lipoprotein fraction III-0 (beta globulin) upon the coagulatory action of the gamma globulin fraction was in marked contrast to that produced by serum albumin. This is shown graphically in Figure 3. Here 2 manifestations of protective action are indicated plainly: (*1*) Progressive inhibition of coagulatory action of gamma globulin with increasing beta to gamma ratios shown in the graph by longer prozones with ratios of 5: 1 and below, and (*2*) suppression of the ability of gamma globulin to induce complete coagulation of the gold sol as shown by the occurrence of intermediate maximum color values when the ratios rose to 10: 1 and above. In this series of graphs, the existence of a critical point (or concentration) beyond which the coagulatory action of gamma globulin suddenly diminishes with decreasing concentration is again apparent. Except for the graph illustrating the results with a beta to gamma ratio of 20: 1, this is seen to occur at a concentration of 0.3 μg. gamma globulin per 2.5 ml. gold sol. The discrepancy shown in the graph of the 20: 1 ratio may be due to technical error since it represents a single determination.

Protective action was also evidenced by fraction IV-1 (alpha globulin) but was somewhat less than that exhibited by beta globulin.

Since fraction IV-3,4 (mixed alpha and beta globulins) contained relatively little lipid as compared with both the fraction labeled alpha globulin and that labeled beta globulin, its protective action

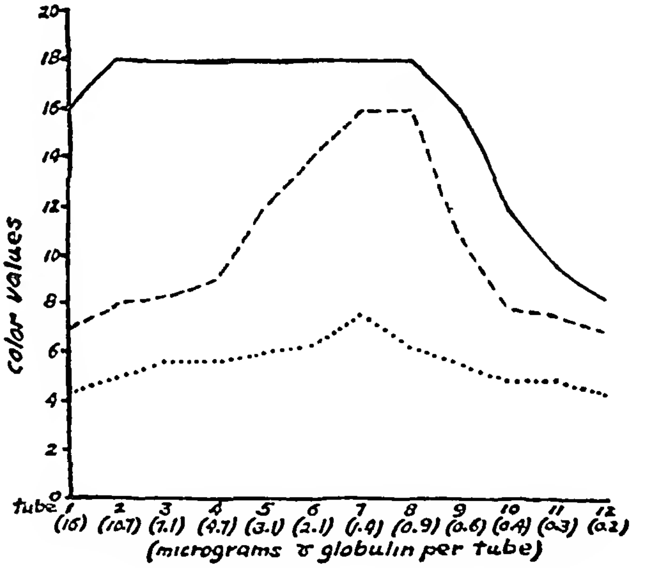

FIG. 4. PROTECTIVE ACTION OF FRACTION IV-3,4 ($\alpha\beta$) (pH 7.4)

Relative proportions: ——— 1.5 $\alpha\beta$: 1 γ, – – – – 3.0 $\alpha\beta$: 1 γ, · · · · · 4.5 $\alpha\beta$: 1 γ.

bumin subjected to repeated salting out with $(NH_4)_2SO_4$ which substantiate this observation—*cf.* C. Lange, Theory of the colloidal gold reaction. I. Reactions between gold sol and isolated protein fractions. J. Lab. and Clin. Med., 1945, 30, 1006.

was investigated and results were obtained as shown in Figure 4. The protein concentrations used for this series of tests were adjusted on the basis of Kjeldahl determinations (N × 6.25). The graphs show an effect similar to that produced by the protective action of the beta globulin fraction but somewhat more pronounced. In addition, the smallest concentration of gamma globulin capable of producing maximum coagulation of the sol in the presence of this fraction was about 3 times greater than that noted in previous experiments. These results appear to indicate that the lipid material bound to the proteins in fractions III-0 and IV-1 played no important role in the protective action of those fractions.

The authors have tried, by combining the fractions in various proportions, to reproduce the curve obtained when diluted normal blood serum (pool of 20 serums) reacts upon a colloidal gold sol. In making these trials it has been assumed that 0.5 ml. of a 1:300 dilution of normal serum contains about 16 μg. of gamma globulin. The normal serum curve shows its maximum, nearly colorless reaction at a dilution of 1:17,000 (about 0.3 μg. gamma globulin). The initial dilution of 1:300 shows but a slight reaction indicating a high degree of protection, while less protection occurs as the dilution increases until the maximum coagulation occurs. In serial dilutions above 1:17,000 the reaction diminishes rapidly. While similar curves have been obtained with some "artificial serums," these all contained greater quantities of the protective proteins than normal serum. In fact, ratios of protective globulin fractions to gamma globulin as high as 3:1 did not show so great protective action as that apparent in normal serum.

DISCUSSION

The experiments reported have been performed in the presence of a uniform hydrogen ion concentration. The authors have unpublished data indicating that blood proteins other than gamma globulin will act as coagulants in the colloidal gold test at pH's on the acid side of their respective isoelectric points and that the coagulant action of gamma globulin is enhanced by lowering the pH. Hence, it is understood that the discussion which follows is predicated upon performance of the test at pH 7.4.

Although it is probable that the gamma globulin fraction used was not composed of a single chemical entity, there seems little doubt, as others have shown, that gamma globulin is the coagulatory protein involved in the colloidal gold reaction of blood serum. The role of serum albumin, however, is not protective; in fact, it is inert toward colloidal gold in a milieu buffered at pH 7.4. The experiments reported leave little doubt that one or more beta globulins (and probably alpha globulins) are the proteins in blood serum which play the major protective role in this reaction. These considerations pose a number of interesting questions which merit investigation by others with technical and clinical facilities which the authors do not have available.

It seems logical to postulate that abnormal colloidal gold reactions arise from either or both of 2 conditions: (*1*) Significant change in the gamma globulin content ot the body fluid and (*2*) alteration in the relative proportions of beta globulin (and possibly alpha globulin) as compared with gamma globulin. Thus, if this theory is true, a spinal fluid giving the typical colloidal gold reaction of general paresis (zone I curve) should contain significantly less of the protective globulins and, in many instances, more gamma globulin than normal spinal fluid. Furthermore, a spinal fluid giving the type of curve associated with the bacterial meningitides (zone III curve) should contain significantly greater quantities of all globulins, particularly of the beta type, than a normal fluid. Reaction patterns associated with certain other conditions, *e.g.*, non-paretic CNS lues (zone II curve), would be explainable on the basis of a moderately altered ratio of the protective globulins to the gamma globulin. Reactions obtained on blood serum in certain types of liver dysfunction are generally of the type which on this basis would be interpreted as arising from a relative decrease in protective globulin content, a hypothesis consistent with the theory that these globulins are elaborated by liver cells while gamma globulin ordinarily is produced elsewhere. The authors are convinced that careful study of the reaction on the basis of these postulates will lead to a more nearly quantitative interpretation of the colloidal gold test.

The utility of the colloidal gold test for the close estimation of gamma globulin content merits fur-

ther careful investigation. The results reported suggest that there is a critical dilution of gamma globulin beyond which complete coagulation of 2.5 ml. of a standardized gold sol does not occur at pH 7.4. With the sols used, this dilution was found to correspond to a concentration in the neighborhood of 0.3 μg. of the gamma globulin fraction. Even in the presence of protective colloids within the range of concentrations normally encountered in body fluids, the highest dilution giving the maximum coagulatory action in a series of dilutions appears to depend upon a critical gamma globulin concentration. In the experiments reported, the critical concentration of gamma globulin in the presence of protective colloids is not so sharply defined as in their absence but the authors feel that more nearly exact knowledge of the actual protein content of the experimental material and greater accuracy in measurements than that afforded by the serial dilution technic are needed before this effect can be judged critically. The experiments indicate that relatively pure gamma globulin fractions may be used to advantage in comparing the sensitivities of different gold sols and in standardizing upon a uniform and reproducible type of sol. Conversely, the possibility of using standardized gold sols in testing the purity of protein fractions seems worthy of further study.

The significance of the fact that various combinations of the several protein fractions failed to reproduce the colloidal gold curve yielded by normal human blood serum cannot be ignored. It is not impossible that some fraction other than those investigated comes into play in the reaction of normal serum, but it is also feasible to assume that alterations in the physical state of the proteins involved may occur, even with newer methods of fractionation, and so influence the properties of their artificial admixture as compared with those of serum.

SUMMARY

Experimental data showing the individual effects of blood fractions on uniformly prepared colloidal gold sols at pH 7.4 are presented which confirm the coagulatory action of gamma globulin and which show that one or more beta globulins (and possibly alpha globulins) are responsible for the protective action of normal body fluids as distinct from the serum albumin which is inert. The use of gamma globulin in determining whether or not 2 batches of gold sol are comparable is suggested. Conversely, a standardized gold sol may be found useful in the analysis of blood fractions. In a discussion of the implications of these results, hypotheses are advanced to account for the usual types of colloidal gold reactions exhibited by body fluids in a milieu buffered at pH 7.4.

BIBLIOGRAPHY

1. Lange, C., Die Ausflockung kolloidalen Goldes durch Zerebrospinalflüssigkeit bei luetischen Affektionen des Zentralnerven-systems. Ztschr. f. Chemotherap. Leipz., 1912, 1, 47.
2. Tiselius, A., Electrophoresis of serum globulin; electrophoretic analysis of normal and immune sera. Biochem. Jour., 1937, 31, 1464.
3. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong S. H., Jr., Chemical, clinical, and immunological studies on the products of human plasma production. I. The characterization of the protein fractions of human plasma. J. Clin. Invest., 1944, 23, 417.
4. Williams, J. W., Petermann, M. L., Colovos, G. C., Goodloe, M. B., Oncley, J. L., and Armstrong, S. H., Jr., Chemical, clinical, and immunological studies on the products of human plasma production. II. Electrophoretic and ultracentrifugal studies of human serum albumin and immune serum globulins. J. Clin. Invest., 1944, 23, 433.
5. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., Taylor, H. L., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J. Am. Chem. Soc., 1946, 68, 459.
6. Gray, S. J., Studies on the mechanism of the spinal fluid colloidal gold reaction. Proc. Soc. Exper. Biol. & Med., 1942, 51, 401.
7. Kabat, E. A., Moore D. H., and Landow, H., An electrophoretic study of the protein components in cerebrospinal fluid and their relationship to the serum proteins. J. Clin. Invest. 1942, 21, 571.
8. Lange, C., Methods for the examination of spinal fluid. Am. J. Syph., Gonor., & Ven. Dis., 1939, 23, 638.
9. Lange, C., and Harris, A. H., The significance of the pH in the gold reaction. J.. Lab. & Clin. Med., 1944, 29, 970.
10. Lange, C., and Harris, A. H., A citrate gold of optimal and reproducible sensitivity for use in the colloidal gold reaction. Its preparation and control. Am. J. Pub. Health, 1944, 34, 1087.

THE EFFECT OF LOCAL COOLING ON THE FILTRATION AND ABSORPTION OF FLUID IN THE HUMAN FOREARM

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Studies relating to the effects of cold on the permeability of human capillaries to protein and fluid have assumed practical importance in recent years because of current interest in refrigeration anesthesia and cryotherapy, as well as because of the casualties caused by exposure to cold during the recent war. Clinical (1) and experimental (2, 3) studies have shown that true frostbite increases the permeability of the capillary wall to both protein and fluid, but less is known quantitatively concerning the immediate effects of temperatures not sufficiently low to freeze the tissues but still injurious enough to produce serious lesions such as "immersion foot" (4).

Lewis showed (5) that immersing the hands in water at various temperatures produced least change in their volume in the range from 15° to 20° C. At 10° C. or less, and at 25° C. or more, their volume increased. After prolonged immersion at 5° C., aspirated edema fluid contained approximately 3 per cent protein. These results suggested strongly that capillary permeability was increased by moderate cold but were not conclusive because the observed changes occurred while venous pressure was elevated and because changes in vascular volume could not be entirely excluded. At about the same time Lange (6) came to the opposite conclusion, viz. that the permeability of cutaneous capillaries in man and the rabbit is decreased by cold because the appearance in the skin of intravenously injected fluorescein was delayed at a temperature of 10° C. However, the rapid diffusibility of this dye makes it a very uncertain indicator of capillary permeability; it seems more likely that reduced blood flow, not decreased permeability, was responsible for the observed delay in staining of the skin.

To obtain more quantitative information, the pressure plethysmograph of Landis and Gibbon (7) was used to measure changes in the volume of extravascular fluid in the forearm during exposure to external temperatures ranging from 44.5° to 4.5° C. It was found that moderate cooling (*a*) increased the extravascular volume of the forearm even when venous pressure was normal, (*b*) decreased the rate of filtration produced by a given increase of venous pressure and (*c*) decreased the rate at which extravascular fluid was reabsorbed. The evidence is consistent with the view that the human capillary wall becomes increasingly permeable to protein and fluid as environmental temperature falls below 25° C., but it also appears that other factors influence the rate at which edema develops during exposure to cold.

METHODS

The pressure plethysmograph of Landis and Gibbon (7, 8) was used throughout. The particular advantage of this method is that the volume of a segment of forearm can be measured very precisely while it is exposed intermittently for brief periods to an external pressure of 200 mm. Hg. This pressure is sufficient to collapse the blood vessels and thus to exclude almost completely the large and unpredictable changes in volume which are caused by vasodilatation or vasoconstriction. By excluding such changes in vascular volume, the accumulation of relatively minute amounts of extravascular fluid could be measured with little error. The effects of temperature could also be studied because the plethysmograph had double walls between which water at any desired temperature could be circulated. The apparatus and general methods used in this study were essentially the same as those described by Landis and Gibbon (7) to whose paper the reader is referred for a detailed description and diagrams.

One important modification in procedure was introduced. Landis and Gibbon (7) began their observations after the subjects had reclined for 30 minutes with the forearm in the plethysmograph. In the observations now described the subjects reclined with the forearm supported in a vertical position for a total of 90 minutes, during the last 30 minutes of which the plethysmograph was being adjusted and filled. This was done in order to insure complete absorption (by the capillaries) or drainage (by the lym-

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phatics) of any extravascular fluid that might have accumulated as a result of previous activity or dependency of the forearm. This prolonged preparation increased the accuracy of the method and also led to modification of certain conclusions reached by Landis and Gibbon (7).

Temperatures were measured at intervals of 5 minutes or less by appropriately placed thermal junctions, viz. in the water surrounding the arm in the plethysmograph, on the skin of the forearm within the plethysmograph, on the skin of the wrist just outside the plethysmograph, and in room air near the plethysmograph. Water from a cool or warm reservoir was circulated at a rate of 5 liters per minute through the space between the inner and outer walls of the plethysmograph and through a jacket surrounding an accessory vessel between the plethysmograph and the burette (7). This kept the water surrounding the arm in the plethysmograph at the desired levels within 1° C. except for transient deviations of not more than 1.5° C., about the average which occurred occasionally as water moved rapidly into or out of the plethysmograph during determinations of volume. For the sake of brevity, only average temperature, *i.e.*, 4.5° for 4° to 5° or 14.5° for 14° to 15°, etc., will be given in the text, it being understood that the range was about plus or minus 0.5° C. as indicated in the charts and tables.

The midpoint of the plethysmograph, with the forearm vertical, was placed level with the manubrium sterni. A pneumatic cuff, 15 cm. in width, was wrapped around the upper arm. Before beginning observations, this was inflated to determine the lowest cuff pressure which would cause an increase in total arm volume. This precaution was taken to avoid possible errors which might arise if the diaphragms of the plethysmograph were applied so snugly that they themselves produced venous congestion. The same cuff was used in certain experiments to produce venous congestion. For this purpose, pressure in the cuff was raised to the desired level plus the additional correction required because of the position of the forearm and the water surrounding it in the plethysmograph (7).

Each determination of "reduced" or "avascular" forearm volume involved application of a pressure of 200 mm. Hg to the water in the plethysmograph for a period of 2 minutes, during which burette readings were made every 30 seconds. Only the last of each set of readings was used to calculate the change in reduced forearm volume that had occurred since the preceding determination. Pressure was usually applied for 2 minutes out of every 10, 8 minutes being allowed for recovery. When venous congestion was imposed between successive determinations, the interval was increased so that the actual time during which filtration occurred and the forearm was not exposed to external pressure was 10 minutes. In certain control observations for special purposes, pressure was applied for 2 minutes out of every 5, 20, or 60 minutes.

At the conclusion of each experiment the upper and lower circumferences of the segment of forearm within the plethysmograph were measured and the approximate volume of the segment was calculated.

Four normal individuals, 3 male and 1 female, aged between 20 and 33, served as subjects throughout. A few supplementary observations were made on another female subject.

OBSERVATIONS

1. *Control studies of "reduced forearm volume" at 34.5° C.*

Landis and Gibbon (7) observed a significant decline in reduced forearm volume during control observations at this and other temperatures. By making few or many determinations over a period of 1 hour or more they concluded that this decline in volume was due chiefly to a real absorption of fluid and only slightly to the mechanical effects of the repeatedly applied external pressure. They omitted, however, the preliminary "emptying" of the forearm which in the present observations reduced the volume of extravascular fluid to a minimum prior to each experiment. When this precaution was taken the existence of a systematic mechanical artefact became clear.

In 2 experiments on each of the 4 regular subjects, reduced forearm volume was measured repeatedly at intervals of 10 minutes for 60 to 80 minutes while the water in the plethysmograph was kept at a neutral temperature, viz. 34.5° C. As shown in Figure 1 (solid dots and solid line) re-

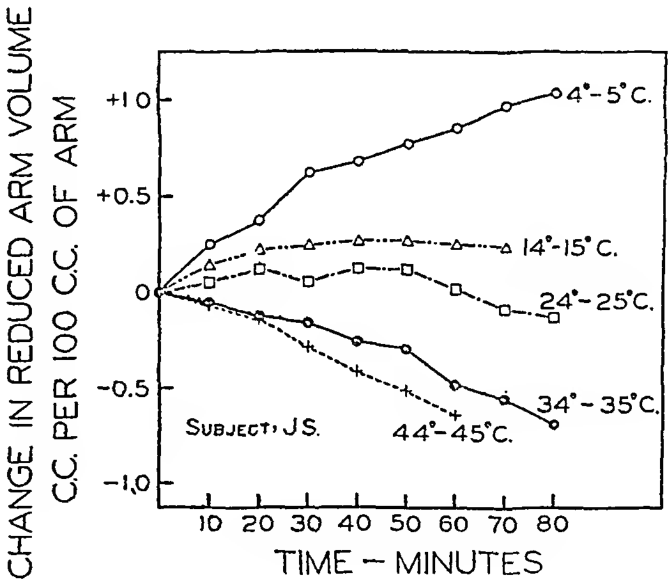

FIG. 1. CHANGES IN REDUCED FOREARM VOLUME, IN ML. PER 100 ML. OF FOREARM, UNDER RESTING CONDITIONS AT PLETHYSMOGRAPH TEMPERATURES OF 4–5°, 14–15°, 24–25°, 34–35°, AND 44–45° C.

A pressure of 200 mm. Hg was applied to the contents of the plethysmograph for 2 minutes out of every 10 for determination of reduced forearm volume. Subject J. S.

duced forearm volume decreased steadily; this was true of all 8 experiments. In 62 measurements of reduced forearm volume the average change per 10-minute period was − .08 ml. per 100 ml. of forearm tissue (range from + .11 to − .33 ml. per 100 ml. of tissue; standard deviation .073). For the 3 male subjects the average change of reduced forearm volume per 10-minute period was between − .06 and − .08 ml. per 100 ml.; for the female subject, − .13 ml. per 100 ml. In 2 similar experiments using a second female subject the average change per 10-minute period was − .16 ml. per 100 ml.

To determine whether this slight but definite decrease in volume was due to true absorption of extravascular fluid or was simply a pressure artefact, serial determinations were made for a period of 1 hour at intervals of 5, 10, and 20 minutes. It was found that the rate of decrease in control volume was related to the number of readings (and number of periods of external pressure) and not to total elapsed time. Thus, over a period of 60 minutes, 12 determinations decreased the volume by an average total of −.79 ml. per 100 ml. of forearm, 6 determinations decreased it by − .55 ml. per 100 ml., and 3 determinations by − .28 ml. per 100 ml. The mean decrease of reduced forearm volume per determination at 5-minute intervals was −.06 ml. per 100 ml., and, at 20-minute intervals, − .10 ml. per 100 ml. Both were within the limits of the probable error of the mean (− .03 to − .13) observed when determinations were made every 10 minutes.

In a single experiment, one pair of determinations 10 minutes apart was separated by a 60-minute interval from another pair of readings 10 minutes apart. Reduced forearm volume did not change between the determinations separated by the interval of 1 hour and the total change in volume in 80 minutes during which pressure was applied 4 times, was only − .15 ml. per 100 ml. Hence, if the forearm is first prepared by reducing excess tissue fluid as much as possible it appears that the gradual decrease in reduced forearm volume at normal venous pressure is due not to true absorption of residual extravascular fluid via the capillaries nor to lymphatic drainage, but to mechanical and progressive squeezing out from the plethysmograph either of the plastic tissues of the forearm or of a slight amount of residual fluid.

The adequacy of the precautions taken in preparing subjects was tested further by 3 experiments in which the amount of extravascular fluid was purposely increased by prior venous congestion with a pneumatic cuff inflated to 55 cm. water for an hour. After the usual 90 minutes of preparation, reduced forearm volume was measured every 10 minutes for an hour. The average decreases in forearm volume per application were − .13 to − .21 ml. per 100 ml., *i.e.*, slightly greater than in the control experiments but still not significantly different because they were still within the 95 per cent probability range of the control experiments (+ .07 to − .23 ml. per 100 ml.).

The 90-minute preparation period was therefore considered sufficiently long to allow for removal from the forearm of such excess fluid as might have been present when the subject first reclined. Because the gradual decrease in volume of the forearm during control observations was apparently a mechanical artefact, the average decrease per application of pressure at 34.5° C. for each subject was used as a correction factor in all quantitative interpretations of the remaining experimental results.

2. *The effects of temperature on reduced forearm volume (or the volume of extravascular fluid) under resting conditions and normal venous pressure*

The temperature of the water in the plethysmograph was constant within the limits given in Table I except for occasional transient excursions of not more than 1° C. beyond these limits during deter-

TABLE I

Relation between the temperatures of the plethysmograph and of the skin of the forearm within the plethysmograph

Number of observations	Plethysmograph temperature*	Skin temperature within plethysmograph	
		Minimum	Maximum
	° C.	° C.	
20	4–5	7.5	14.5†
10	14–15	13.5	21.0‡
10	24–25	24.5	28.5
20	34–35	33.5	35.5
7	44–45	40.0	43.5

* Transient deviations ±1.0° C. beyond indicated range occurred occasionally.
† 80 per cent between 8.5° and 12.5° C.
‡ 80 per cent between 14.0° and 19.5° C.

minations of volume. At plethysmograph temperatures above or below 34.5° C. the skin within the plethysmograph was rarely at the same temperature as the water because (*a*) a thin sheet of rubber separated the water from the skin, and (*b*) skin temperature was affected by blood flow, which was not interrupted except during the brief periods when external pressure was applied. Hence, as shown in Table I, during exposure to water at 4.5° C. skin temperatures were usually between 8.5° and 12.5° C. (This was about as much cooling as could be tolerated without unbearable pain over the 1½ hours required for a complete observation.) The temperatures of the skin and the plethysmograph were most nearly equal at 34.5° C., which can be regarded therefore as a close approach to "neutral" temperature.

The extent to which the forearm was exposed to cooling during the 30 minutes prior to beginning observations varied somewhat depending upon the ease or difficulty of the preliminary adjustments. Room temperatures ranged from 20° to 29.5° C. and, with one exception, changed not more than 2.5° C. during any experiment.

Figure 1 illustrates for one subject the changes in reduced forearm volume when measured at 10-minute intervals during continuous exposure of the uncongested arm to 5 temperatures ranging from 4.5° to 44.5° C. for periods of an hour or longer. At 4.5° C., the volume of the forearm increased rapidly at first and then more slowly; a similar but less marked increase in volume occurred at 14.5° C. It has been mentioned that the apparent decrease at 34.5° C. is an artefact so that, in absolute terms, volume increased slightly even at 24.5° C.

Table II summarizes the results obtained in 4 subjects; observed and, for quantitative comparison, also the corrected figures are given. The latter were obtained by adding to each observed reduced forearm volume the total mechanical decrease in volume expected to result from 6 applications of pressure at 34.5° C. (6 times the average decrease per application of external pressure at 10-minute intervals as determined for the same subject in the control experiments).[4] The average increase in volume of extravascular fluid at 14.5° C. was almost double and at 4.5°C. almost triple that at 24.5° C. Only small and inconsistent changes occurred at 44.5° C. It seems clear, however, that the regular and progressive change in volume between 4.5° C. and 34.5° C. is arrested or possibly reversed at some temperature between 34.5° and 44.5° C. The changes resulting from cooling were largest in E. B. and least in C. W.

[4] To test the assumption that the pressure artefact was the same at low temperatures as at 34.5° C., 2 experiments were done at 4.5° C. in which 2 pairs of determinations 10 minutes apart were separated by an interval of 60 minutes. In each case the total increase in reduced forearm volume during 60 minutes of exposure to cold without applications of external pressure was considerably greater than that observed during exposure to cold for the same time when readings were made in routine fashion every 10 minutes. The validity of the assumption was also demonstrated quantitatively because the difference in volume at the end of 60 minutes of cooling with and without readings at 10-minute intervals was approximately equal to that expected from 6 applications of external pressure at 34.5° C.

TABLE II

Total change in reduced forearm volume (observed and corrected) during 60 minutes at temperatures from 4° C. to 45° C.

Subject	Change in volume, *ml. per 100 ml. of forearm*									
	4 to 5° C.		14 to 15° C.		24 to 25° C.		34 to 35° C.		44 to 45° C.	
	Observed	Corrected	Observed	Corrected	Observed	Corrected	Observed	Corrected	Observed	Corrected
J. S.	+ .56*	+1.02	+.26	+ .72	+.01	+.47	−.46†	0	−.64	−.18
C. W.	+ .21*	+ .67	−.05	+ .41	−.14	+.32	−.46†	0	−.23	+.23
E. W.	+ .30*	+ .67	+.37	+ .74	+.06	+.43	−.37†	0	−.46	−.09
E. B.	+1.04*	+1.79	+.27	+1.02	−.40	+.35	−.75†	0	−.30	+.45
Average (corrected)		+1.04		+ .72		+.39		0		+.10

* Average of 2 experiments.
† Standard of reference and value used as correction figure. Six times the average change in reduced forearm volume per determination when an external pressure of 200 mm. Hg was applied for 2 minutes out of every 10 minutes.

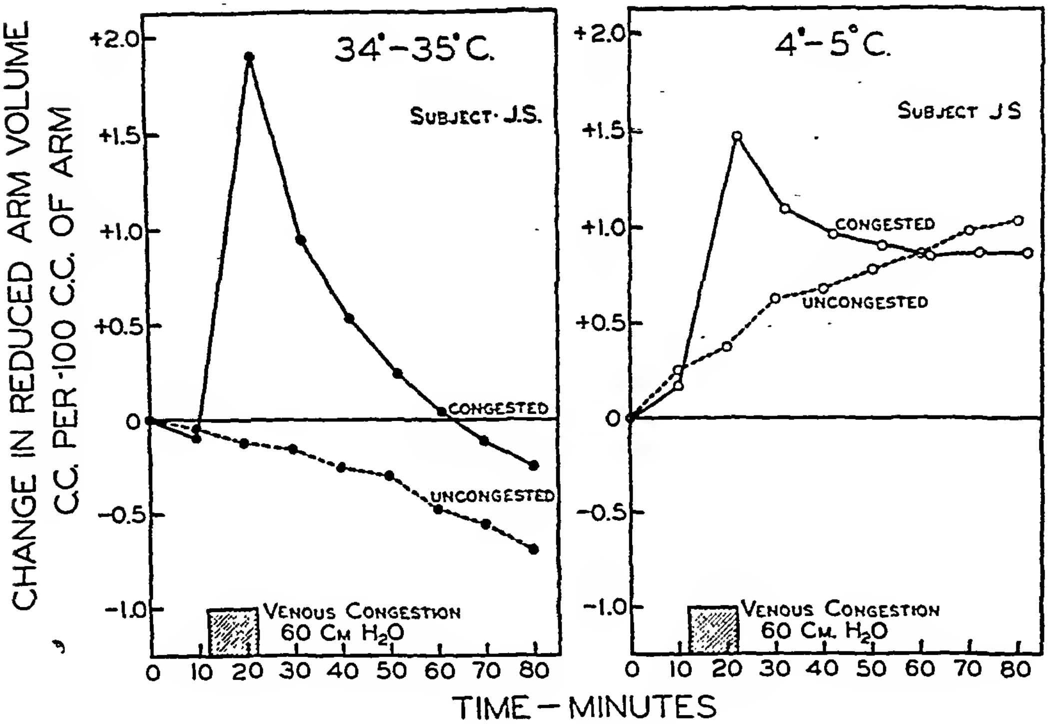

FIG. 2. CHANGES IN REDUCED FOREARM VOLUME, IN ML. PER 100 ML. OF FOREARM DURING AND AFTER A 10-MINUTE PERIOD OF CONGESTION AT 60 CM. WATER VENOUS PRESSURE. PLETHYSMOGRAPH TEMPERATURES 34–35° AND 4–5° C.

Changes in reduced forearm volume under resting conditions at the same temperatures (see Figure 1) are indicated by dotted lines. Subject J. S.

and E. W.; this relation was fairly consistent throughout the whole series suggesting a real difference between individual subjects.

3. The effect of temperature on the relation between venous pressure and filtration of fluid

Landis and Gibbon (7) observed that during congestion at a given venous pressure, *e.g.* 60 cm. water, for 10 minutes, the volume of fluid which was filtered was greatest at 44° to 45° C. and least at 14° to 15° C., but they did not test the effect of still lower temperatures. Hence these observations were extended by experiments similar to those shown in Figure 2.

After the usual preparation (totaling 90 minutes) 2 determinations of reduced forearm volume were made, 10 minutes apart. As external pressure was released after making the second reading, venous pressure was raised abruptly to 20, 40, or 60 cm. water by inflating the pneumatic cuff around the upper arm. Venous congestion was continued for 10 minutes; the cuff was then deflated and reduced forearm volume was measured immediately. Determinations of reduced forearm volume were then repeated at intervals of 10 minutes for totals of 40 to 60 minutes in order to measure the rate at which the recently filtered fluid was reabsorbed or conducted away by the lymphatics.

At 34.5° C. (solid line, Figure 2, left) the observed increase in reduced forearm volume resulting from 10 minutes of congestion at a venous pressure of 60 cm. water was almost 2.0 ml. per 100 ml. of forearm. After release of the congesting cuff, the volume of excess tissue fluid which had accumulated as a result of congestion decreased rapidly. For comparison, the changes in reduced forearm volume which took place in the same subject at 34.5° C. when no congestion was applied are also shown (dotted line, Figure 2, left). At 4.5° C., the effects of a similar period of congestion differed as follows: (*a*) reduced forearm volume began to increase even before venous pressure was elevated, (*b*) the total volume of fluid filtered dur-

ing congestion was less than at 34.5° C. rather than more as might have been expected if the only effect of cold were an increase in capillary permeability, (*c*) the rate of removal of excess fluid after release of the congesting cuff was slower, and (*d*) reabsorption stopped when the volume of excess tissue fluid was approximately equal to that present after exposing the arm to a temperature of 4.5° C. for the same time without congestion (dotted line, Figure 2, right).

The results pertaining to filtration in a series of such experiments are summarized in Table III, in which all figures have been corrected to exclude the pressure artefact. In agreement with Landis and Gibbon (7), the volume of fluid filtered was always greater the higher the venous pressure at any given temperature. When venous pressure was 60 cm. water the average rate of filtration was greatest at 34.5° C., least at 14.5° C., and then rose again to an intermediate value at 4.5° C. At a venous pressure of 40 cm. water, average filtration at 4.5° C. was clearly less than that at 34.5° C., but the difference was not striking. At a venous pressure of

TABLE III

Effect of temperature of the plethysmograph on rate of filtration of fluid into the tissues of the forearm during 10-minute periods of venous congestion

Congesting pressure	Subject	Rate of filtration of fluid*				
		4 to 5° C.	14 to 15° C.	24 to 25° C.	34 to 35° C.	44 to 45° C.
cm. H_2O		*ml. per 100 ml. per min.*	*ml. per 100 ml. per min.*	*ml. per 100 ml. per min.*	*ml. per 100 ml. per min.*	*ml. per 100 ml. per min.*
20	J. S.	.063	.053	.074	.050	.058
	C. W.	.036			.021	
	E. W.	.039			.041	
	E. B.	.063			.064	
	Average	.050			.044	
40	J. S.	.099	.107	.106	.121	.118
	C. W.	.065			.067	
	E. W.	.096			.111	
	E. B.	.102			.137	
	Average	.091			.109	
60	J. S.	.138	.152	.185	.208	.226
	C. W.	.093	.096	.093	.115	
	E. W.	.173	.151	.147	.152	
	E. B.	.147	.108	.184	.158	
	Average	.130	.102	.152	.158	

* All values corrected to exclude pressure artefact.

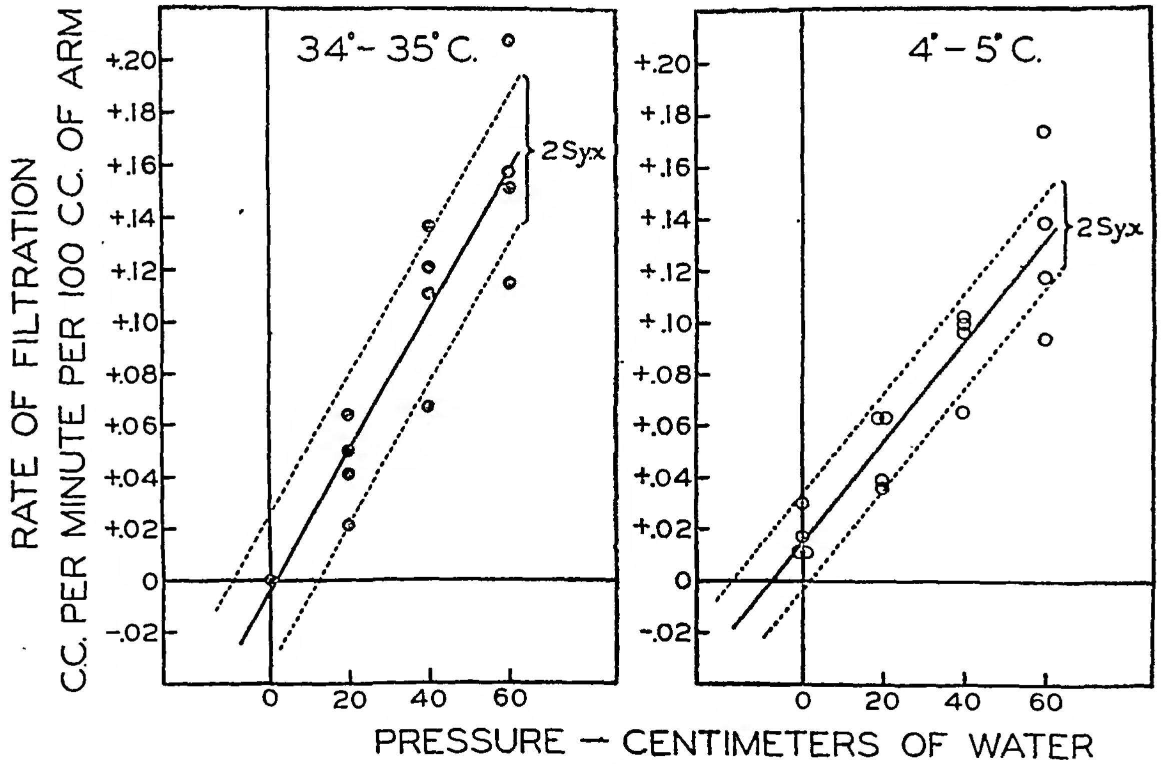

FIG. 3. RATE OF FILTRATION, IN ML. PER MINUTE PER 100 ML. OF FOREARM DURING 10-MINUTE PERIODS OF VENOUS CONGESTION WITH CUFF PRESSURES OF 20, 40, AND 60 CM. WATER. PLETHYSMOGRAPH TEMPERATURES 34–35° AND 4–5° C.

Changes in reduced forearm volume under resting conditions appear as filtrations at zero congesting pressure at 4–5° C. Four subjects. Results corrected to exclude pressure artefact.

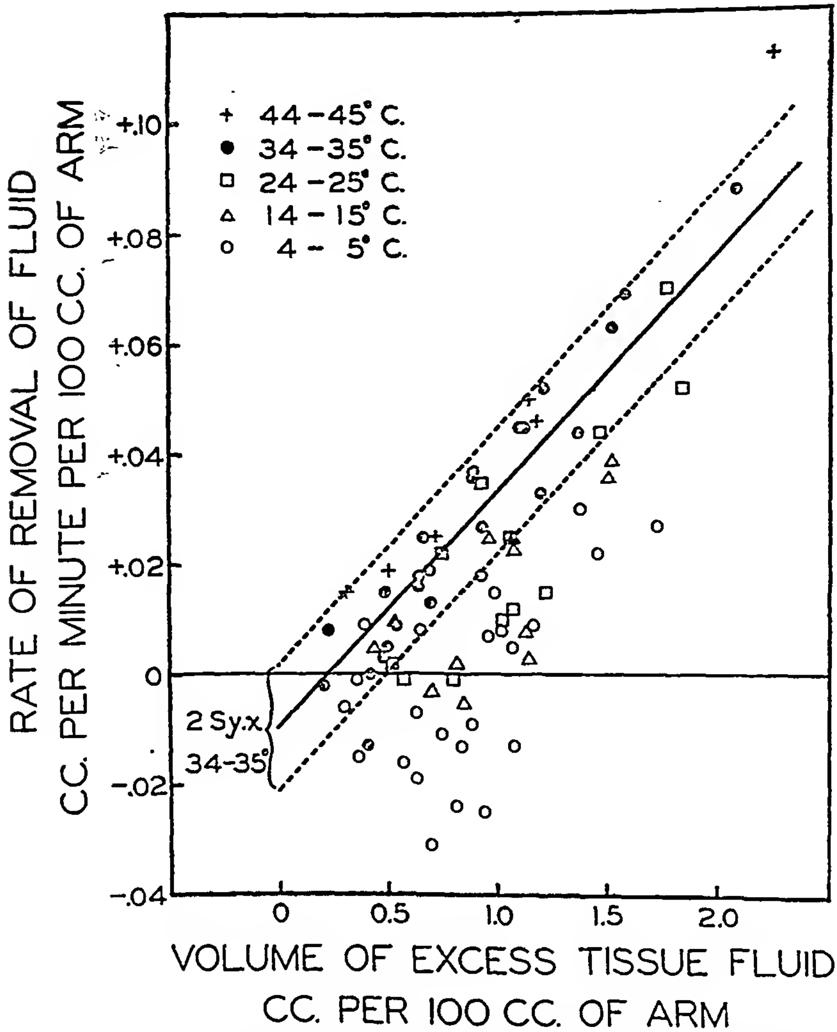

FIG. 4. RELATION BETWEEN THE VOLUME OF EXCESS TISSUE FLUID PRESENT, IN ML. PER 100 ML. OF FOREARM, AND THE RATE OF REABSORPTION OF FLUID, IN ML. PER MINUTE PER 100 ML. OF FOREARM. PLETHYSMOGRAPH TEMPERATURES 4–5°, 14–15°, 24–25°, 34–35°, AND 44–45° C.

The volume of excess tissue fluid was obtained from the first 2 observations of reduced forearm volume after 10 minute periods of congestion at venous pressures of 20, 40, and 60 cm. water. Four subjects. Results corrected to exclude pressure artefact.

20 cm. water the filtration rates were approximately the same at 4.5° and 34.5° C. because (*a*) at this relatively low venous pressure the effects of venous congestion were complicated by the swelling described above for the uncongested arm (Figure 1), and (*b*) the error for any single measurement was relatively great when small volumes of filtrate were involved.

The linear relation between rate of filtration and grade of venous congestion is shown for 34.5° C. and for 4.5° C. in Figure 3. Reasons have been given above for believing that after prolonged preparation of the forearm and at a temperature of 34.5° C. the corrected, reduced forearm volume is practically constant. Hence, all 4 points relating to the resting forearm (zero congesting pressure) at 34.5° C. have been placed at zero filtration (Figure 3, left). At this temperature filtration began as soon as venous pressure was raised above the resting level and increased linearly with increasing grades of venous congestion. At 4.5° C., filtration occurred at a rate of about .02 ml. per minute per

100 ml. of forearm even at zero congesting pressure. At this temperature filtration also increased with increasing congesting pressure, but less rapidly than at 34.5° C.

The slopes of these lines permit estimation of the unit rate of filtration, *i.e.,* the rate of filtration resulting from each centimeter rise of venous pressure, at each temperature. At 34.5° C. the unit rate of filtration was .0027 ml. per minute per 100 ml. of forearm per cm. venous pressure (standard deviation .00025); at 4.5° C., it was .0019 ml. per minute per 100 ml. of forearm per cm. venous pressure (standard deviation .00020). The chances are better than 3 out of 4 that this reduction of 30 per cent in the unit rate of filtration was not due to errors of random sampling (k 1.18).

It is noteworthy that at 4.5° C. the extension of the mean line crosses the line of zero filtration at a pressure of − 9 cm. water. The shift of the intercept toward the left (with respect to the intercept of the line fitting points obtained at 34.5° C.) is consistent with the view that cold (4.5° C.) increased the permeability of the capillary walls to protein and that the swelling of the forearm at normal venous pressure (Figure 1) was due to a reduction of the effective colloid osmotic pressure of the blood amounting to approximately 9 cm. water.

4. The effect of temperature on the rate at which recently filtered extravascular fluid is reabsorbed after congestion

Landis and Gibbon (7) observed that small amounts of excess tissue fluid sometimes were removed from the forearm less rapidly during cooling (14° to 15° C.) than at higher temperatures. This effect of cold is far more striking at 4.5° than at 14.5° C., as shown in Figure 4 where the rate of removal of fluid is charted against the volume of excess tissue fluid present at the time reabsorption was measured. These values were obtained from measurements of reduced forearm volume 10 minutes and 20 minutes after release of the congesting cuff in experiments similar to those illustrated in Figure 2. At each of the 5 temperatures designated, the rate at which fluid was removed was greater when larger volumes of excess tissue fluid were present because tissue pressure was raised by the accumulated fluid (7). At 34.5° C., a linear relationship became clear (correlation coefficient + .88); at other temperatures the points were more scattered. However, for any given volume of excess tissue fluid, the rate of reabsorption was clearly least at 4.5° C. In agreement with earlier results (7) the rates of removal at 14.5° C. were not always reduced and the difference was less conspicuous. The results at 24.5° and 44.5° C. were so nearly within the zone of results obtained at 34.5° C. that conclusions are not warranted.

The marked effect of cold (4.5° C.) on reabsorption of tissue fluid and the importance of this factor in "immersion foot" are shown more graphically in Figure 2. At 34.5° C., reabsorption was almost complete within 60 minutes after the end of the period of congestion; at 4.5° C. reabsorption was slower and remained incomplete, less than half of the filtered fluid having been absorbed at the end of 60 minutes. At 34.5° C., in 4 subjects, between .43 and 1.19 ml. of fluid were removed per 100 ml. of forearm tissue during the first 30 minutes after a prior congestion at 60 cm. water for 10 minutes. At 4.5° C. during a similar period only .10 and .32 ml. of fluid were removed in 2 subjects and in 2 others the volume of tissue fluid increased by .05 and .59 ml. per 100 ml. of forearm tissue. This disturbance of fluid removal is compatible with a reduction of the effective colloid osmotic pressure of the plasma amounting to approximately 10 cm. water.

DISCUSSION

Determination of the volume of tissue fluid by means of the pressure plethysmograph was made more accurate by having the subject recline with the forearm supported in a vertical position for 90 minutes before observations were begun. This period of preparation permitted capillary absorption and lymphatic drainage to evacuate any excess tissue fluid that had accumulated during immediately prior activity or dependency so that equilibrium was established between the circulating blood and the volume of tissue fluid. Landis and Gibbon (7) did not use this precaution and concluded that successive applications of external pressure did not *per se* change limb volume consistently and that the decrease in volume was due to continued absorption. It appears now that they observed this transitory absorption because the

forearm contained tissue fluid remaining from previous activity or dependency. If excess tissue fluid has been removed by suitable preparation, neither filtration nor absorption occurs at 34.5° C. when the forearm is placed at heart level in the plethysmograph, and the importance of a small and uniform correction for a pressure artefact becomes clear.

The correction for this artefact differed even in normal subjects, being almost twice as great in 2 female subjects as in 3 male subjects. This may be related to differing proportions of muscle, subcutaneous tissue, and skin in their forearms; but the possibility that small amounts of tissue fluid, or even intracellular fluid, are expressed from the regions near the ends of the plethysmograph cannot be excluded. In any case it is important to eliminate any differences in the volume of tissue fluid which may be present in the forearm prior to each experiment and to determine the pressure artefact for each subject, especially if patients with edema are to be studied.

This small correction was relatively unimportant when large volumes of fluid were filtered (*e.g.* Figure 2 and Table III) but became very important in measuring fluid movement at normal venous pressure and in locating the lowest venous pressure at which filtration began. Thus, without special preparation of the forearm, Landis and Gibbon found that at 34° to 35° C. venous pressure had to be raised by 10 to 17 cm. water before a definite increase in the uncorrected volume of the forearm could be produced. With proper preparation and correction for the pressure artefact the line relating venous pressure and filtration passes very close to zero, which is more nearly in accord with the Starling hypothesis (Figure 3, left).

It seems quite clear that cold can produce significant filtration of fluid at resting venous pressure (Figures 1, 2, and 3). This is consistent with direct or indirect injury of the capillary wall, increased capillary permeability to protein, and lowering of the effective colloid osmotic pressure of the plasma. The changes actually observed during cooling of the plethysmograph to a given temperature corresponded to smaller changes of tissue temperature because (*a*) the temperature of the skin was always somewhat higher than that of the water in the plethysmograph (Table I), and (*b*) deep tissue temperature was even higher than skin temperature (9). Yet slight changes were observed when the plethysmograph was cooled to only 24.5° C. and when skin temperature was only 5 degrees below the levels usually found when exposed to comfortable environmental air. The effects became progressively more severe as temperature was reduced further. It was not possible to find any critical temperature at which a great increase in filtration appeared abruptly. Table II suggests that at some plethysmograph temperature between 34.5° and 44.5° C. (skin temperature between 34.5° and 42° C.), the volume of the forearm remained most nearly constant. Above and below that temperature the volume of tissue fluid increased, on the average, even while venous pressure was normal.

The possibility must be considered that the increase in extravascular volume of the uncongested forearm during exposure to cold was due to swelling of cells and not to accumulation of intercellular fluid. This was probably not the case for several reasons. In the first place such increase occurred at rates characteristic of fluid movement through the capillary wall which has a permeability to fluid between 100 and 3000 times greater than that of a typical cell membrane (10). At 4.5° C., fluid accumulated on the average at a rate of .017 ml. per 100 ml. of forearm tissue per minute. Continued accumulation at this rate would produce definite pitting edema in about 10 hours, though developing tissue pressure would decrease the rate of filtration and thus make this period considerably longer. Second, fluid known to be filtered during venous congestion was not reabsorbed fully so that at the end of 80 minutes at 4.5° C. the extravascular volume after congestion and partial recovery tended to equal that of the uncongested arm after the same period of exposure (Figure 2, right). Third, Lewis (5) was able to obtain several samples of edema fluid containing approximately 3 per cent protein by inserting needles into the subcutaneous tissues of the cooled hand.

Other things being constant, it would be expected that capillary injury would lead not only to filtration of fluid at normal venous pressure, but also to conspicuous increase of filtration rates observed during venous congestion. While the former was observed (Figure 1, Table II), the latter was not (Figure 3).

This may have been due, in part, to the necessity of exposing the forearm to some cooling during the period of 30 minutes required for adjusting the plethysmograph. Any fluid filtered at resting venous pressure during this time would raise tissue pressure and thereby impede further filtration. It is also possible that increased viscosity of the filtered fluid may have affected filtration, but the differences were greater than would be expected on this basis alone. Of more importance, however, were the concomitant effects of temperature on blood vessels and blood flow in the cooled forearm.

Even while cold was beginning to increase capillary permeability, it was also producing arteriolar constriction, reducing blood flow (9), reducing the gradient of capillary pressure (11), and probably reducing the area of capillary wall available for continued filtration by stopping blood flow in some vessels entirely. Recent studies (9) have shown that blood flow in the forearm is about 17.6 ml. per 100 ml. of tissue per minute at 45°, 4.3 ml. at 35°, 0.7 ml. at 25°, and 0.5 ml. at 10 to 15° C. Blood flow in the hand is affected similarly except for an increase below 15° C. (12), but this reactive hyperemia has been ascribed primarily to the opening of arteriovenous anastomoses in the finger tips (13). Incomplete studies (14) have shown that the collective volume of the blood vessels, *i.e.*, the vascular volume, of the forearm is reduced to ½ by cooling from 34° to 24° C. and to ⅓ by cooling from 34° to 14° C. Unfortunately, exact figures are not available for lower temperatures but there is little reason to expect an increase in either blood flow or vascular volume in the forearm at skin temperatures below 10° to 15° C. because there are few arteriovenous anastomoses in this region.

It should be noted that such a decrease in blood flow would be expected to reduce the rate at which a diffusible dye such as fluorescein would enter the cooled skin. The results of Lange (6) can therefore be explained on the basis of diminished transport of dye to the cooled tissues and do not indicate that capillary permeability is decreased by cold. In similar fashion the decreased unit rate of filtration observed during exposure to cold was probably a secondary effect of vasoconstriction and hence not evidence against an increase of capillary permeability to protein.

The degree to which permeability to protein was increased by cold can be estimated in terms of the amount by which the effective colloid osmotic pressure of the plasma was reduced, as shown by the observed filtrations and absorptions. During the most extreme cooling, extravascular fluid accumulated in the uncongested forearm at an average rate of .017 ml. per minute per 100 ml. of tissue which, according to Figure 3, left, is equivalent to the effect of increasing venous pressure (or decreasing the effective colloid osmotic pressure) by 7.5 cm. water. Comparison of the intercepts in Figure 3 indicates that effective colloid osmotic pressure was reduced by approximately 9 cm. water at 4.5° C. The delayed absorption at 4.5° C. shown in Figure 4 is consistent with a reduction of effective colloid osmotic pressure amounting to approximately 10 cm. water. The effective colloid osmotic pressure of the plasma would be lowered as much as this (7 to 10 cm. water) by the passage of a capillary filtrate containing 2 per cent protein (15) rather than the normal 0.3 per cent or less (16).

Efforts to substantiate this conclusion by determining the concentration of plasma protein in capillary filtrate by a modification of the method described by Landis *et al* (16) were unsuccessful; values obtained varied too widely to be conclusive. It may be recalled, however, that Lewis found approximately 3 per cent protein in edema fluid aspirated from the tissues of the hand after prolonged exposure to cold. Despite possible contamination of these samples by blood plasma from mechanical injury or rupture of capillaries, these figures agree well with those calculated from plethysmographic measurements of filtration and absorption at low temperatures.

In contrast to these effects in man it is noteworthy that Brown and Landis (17) found that the frog's capillaries were affected quite differently by cold as is, in fact, necessary for the survival of a poikilothermic animal, exposed for long periods to very low temperatures. When local temperature was decreased 20° C. the permeability of the walls of single capillaries in the frog's mesentery decreased 73 per cent and there was a significant increase in the total effective osmotic pressure of the blood. The cause for the latter is not clear although thermosmosis or the development of a transient effective osmotic pressure by

plasma constituents other than proteins are possible explanations that need further study. If either of these factors plays any part in man, the results are unrecognizable because the injurious effects of cold produce more conspicuous changes in the opposite direction.

In the human being, the effect of cold on the balance between blood and tissue fluid is chiefly the result of interplay between capillary injury and reduction of filtering (and absorbing) area. The first, by permitting the passage of protein, reduces the effective colloid osmotic pressure of the blood and favors the formation of edema even when venous pressure is normal. It also prevents the reabsorption of filtered fluid. The second reduces or delays, but does not prevent, filtration when venous pressure is raised as in a dependent extremity. It also delays conspicuously the reabsorption of such tissue fluid as has been filtered previously. If temperature becomes sufficiently low, the edema-producing effects of injury may exceed the edema-preventing factors related to lessened filtering area and reduced blood flow. In Table III, it is shown that filtration during congestion at 60 cm. water declined from 34.5° to 14.5° C. but rose sharply again at 4.5° C. The appearance of striking edema in "immersion foot" (4) is explicable on this basis because the syndrome appears when the dependent extremities are exposed for brief periods to very low temperatures or for longer periods to temperatures which are only moderately low.

SUMMARY

1. The pressure plethysmograph was used to measure changes in the extravascular ("reduced") volume of the forearm in 4 normal human subjects, in order to determine the effects of local cooling on (*a*) the volume of tissue fluid under resting conditions, (*b*) the rate of filtration of fluid per unit increase of venous pressure and (*c*) the rate of reabsorption of excess tissue fluid after release of venous congestion.

2. The accuracy of this method for measuring minute changes in the volume of tissue fluid has been improved (*a*) by elevating the forearm for 90 minutes to remove residual tissue fluid before each observation, and (*b*) by measuring for each subject the small error or artefact introduced each time a volume is read while applying to the forearm an external pressure of 200 mm. Hg.

3. Cold produced filtration of fluid and increased the volume of tissue fluid even in the uncongested forearm. The average rate of filtration at 4.5° C. was .017 ml. per 100 ml. of forearm tissue per minute. Smaller rates of filtration were observed at 14.5° and 24.5° C. At 34.5° and 44.5° C. reduced forearm volume did not change significantly.

4. The rate of filtration remained directly proportional to the increase in venous pressure even at 4.5° C., but the unit rate of filtration (ml. fluid per 100 ml. of forearm per minute per cm. rise of venous pressure) was 30 per cent less at 4.5° C. than at 34.5° C.

5. Cold reduced the rate of reabsorption of previously filtered fluid conspicuously at 4.5° C. and slightly at 14.5° C.

6. The results collectively indicate that cold increases the permeability of the human capillary wall to protein. In the forearm exposed to water at 4.5° C., this reduces the effective colloid osmotic pressure of the blood by 7 to 10 cm. water.

7. The relation between these findings and the pathogenesis of edema in extremities exposed to cold, *e.g.* "immersion foot," is discussed.

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BIBLIOGRAPHY

1. Davis, L., Scarff, J. E., Rogers, N., and Dickinson, M., High altitude frostbite. Preliminary report. Surg., Gynec. & Obst., 1943, 77, 561.
2. Lewis, T., and Love, W. S., Vascular reactions of the skin to injury. III. Some effects of freezing, of cooling and of warming. Heart, 1926, 13, 27.
3. Greene, R., The immediate vascular changes in true frostbite. J. Path. & Bact., 1943, 55, 259.
4. Ungley, C. C., Channell, G. D., and Richards, R. L., The immersion foot syndrome. Brit. J. Surg., 1945, 33, 17.
5. Lewis, T., Swelling of the human limbs in response to immersion in cold water. Clin. Sci., 1939, 4, 349.
6. Lange, K., The effect of cold on capillary permeability, preliminary report. Bull. New York M. Coll., 1942, 5, 154.
7. Landis, E. M., and Gibbon, J. H., The effects of temperature and of tissue pressure on the movement of fluid through the human capillary wall. J. Clin. Invest., 1933, 12, 105.

8. Krogh, A., Landis, E. M., and Turner, A. H., The movement of fluid through the human capillary wall in relation to venous pressure and to the colloid osmotic pressure of the blood. J. Clin. Invest., 1932, 11, 63.
9. Barcroft, H., and Edholm, O. G., The effect of temperature on blood flow and deep temperature in the human forearm. J. Physiol., 1943, 102, 5.
10. Landis, E. M., Capillary pressure and capillary permeability. Physiol. Rev., 1934, 14, 404.
11. Landis, E. M., Micro-injection studies of capillary blood pressure in human skin. Heart, 1930, 15, 209.
12. Spealman, C. R., Effect of ambient air temperature and of hand temperature on blood flow in the hand. Am. J. Physiol, 1945, 145, 218.
13. Grant, R. T., and Bland, E. F., Observations on arteriovenous anastomoses in human skin and in the bird's foot with special reference to the reaction to cold. Heart, 1931, 15, 385.
14. McLennan, C. E., and Landis, E. M., Unpublished observations.
15. Wies, C. H., and Peters, J. P., The osmotic pressure of proteins in whole serum. J. Clin. Invest., 1937, 16, 93.
16. Landis, E. M., Jonas, L., Angevine, M., and Erb, W., The passage of fluid and protein through the human capillary wall during venous congestion. J. Clin. Invest., 1932, 11, 717.
17. Brown, E., and Landis, E. M., Effect of local cooling on fluid movement, effective osmotic pressure and capillary permeability in the frog's mesentery. Amer. J. Physiol., 1947, 149, 302.

ABNORMAL RENAL TUBULAR BACK-DIFFUSION FOLLOWING ANURIA

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.ive value for $T_{m\text{p-aminohippurate}}$ was ob- a patient with sulfathiazole intoxication 4 days of anuria and 5 days of marked This negative T_{mPAH} is interpreted as in- not only that the tubule cells had lost their .o excrete PAH but that a portion of the iltered by the glomeruli was escaping from ›ular urine by back-diffusion. It is the pur- e of this paper to describe quantitatively the ›rn of glomerular and tubular functional ;es following the period of anuria to apparent functional recovery.

On March 1, 1945 an 8-year-old white female developed sore throat and fever. There was no history of previous renal disease. She received approximately 3.0 grams of sulfathiazole in the form of chewing gum from March 3 to March 6. On March 6, vomiting occurred and continued for 10 days; March 7, anuria appeared and persisted for 4 days, followed by marked oliguria for 5 days. On the third day of anuria, a discrete, macular, erythematous rash appeared on all 4 extremities, and extended during the next 2 days to involve the face and become confluent on the upper extremities. On the fourth day of anuria, bilateral conjunctivitis appeared. The rash and conjunctivitis were present for 10 days. Systolic blood pressure ranged from 130 to 120 mm. and the diastolic from 96 to 80 mm. Hg from the fifth to the ninth day following onset of anuria. Three weeks later, the blood pressure was 110/64 mm. Blood non-protein nitrogen was 214 mgm. per cent 5 days following the onset of anuria and fell gradually to 21 mgm. per cent over a period of 3 weeks. Carbon dioxide combining power was 48 and 57 volumes per cent on the sixth and eighth days following the onset of anuria. Plasma chlorides rose from 71 milliequivalents to 104 in 3 weeks. The red blood cell count, hemoglobin and plasma proteins were normal. Four days following onset of anuria, the patient voided 15 ml. of urine in which a trace of protein was present, red and white cells were absent, and sulfathiazole crystals were seen; the plasma level of sulfathiazole at this time was 2 mgm. per cent. On the seventh and eighth days following onset of anuria, the urine contained moderate amounts of protein, 20 to 50 red blood cells per high power field, and innumerable white blood cells. In 3 weeks, the urine contained no protein or cellular elements. Spontaneous diuresis occurred on the tenth day following the onset of anuria and apparently was not related to any specific therapeutic measure.

The patient was discharged from the hospital 39 days after the onset of anuria, and has been observed for a period of 92.5 weeks following the onset of anuria at the Nephritis and Hypertension Clinic, New York University Clinic. She has attended the clinic at regular intervals and has been symptom free; edema has been absent and the blood pressure has varied from 110/74 to 92/66 mm. Hg; proteinuria reappeared and has persisted, varying from a trace to 2 plus. Hematuria and pyuria have been absent.

METHODS

The rate of glomerular filtration, the effective renal plasma flow, and the maximal tubular excretory capacity (T_{mPAH}) were measured according to the technique described by Goldring and Chasis (1).[1] Intravenous infusion for maintaining plasma levels and bladder catheterization to insure complete bladder emptying were employed.

RESULTS

The results of 5 determinations of specific renal functions are presented in Table I. Two weeks after the onset of anuria, the mannitol and p-aminohippurate clearances were markedly below the

TABLE I

Time from onset of anuria	Plasma clearance*		T_{mPAH}	Filtration fraction (C_m/C_{PAH})	Proteinuria
	Mannitol (C_m)	PAH (C_{PAH})			
weeks	*ml. per minute*		*mgm. per min.*	*per cent*	
2.0	17.2	14.6	−1.1		Trace
3.5	75.0	220.4	−9.4	34.0	None
4.5	79.6	443.0	20.3	17.7	None
7.0	108.4	448.5	42.7	24.2	Trace
92.5	91.0	586.0	85.6	15.6	Two plus

* All clearance values corrected to 1.73 square meters surface area.

[1] Clearances corrected as suggested by Barker and Clark (2).

mean normal values for females (117 and 594 ml. per minute, respectively) and Tm_{PAH} had a negative value of -1.1 mgm. per minute as compared to the normal value of 82.2 mgm. per minute (3). This observation was made 1 week after spontaneous diuresis had occurred and while the blood urea was 93 mgm. per cent.

Three and one-half weeks after the onset of anuria, the mannitol and p-aminohippurate clearances had increased significantly, while Tm_{PAH} continued to have a negative value.

Four and one-half weeks and 7 weeks after the onset of anuria, the mannitol clearance had returned to the normal range, the p-aminohippurate clearance was aproaching normal, and Tm_{PAH} had increased to significant positive values. The blood urea at this time was normal and the urine contained no protein or abnormal cellular elements.

Ninety-two and one-half weeks after the onset of anuria, the above renal functions were in the normal range. The urine contained a moderate amount of protein.

DISCUSSION

Skin rash, conjunctivitis, anuria, sulfonamide crystalluria, and sulfonamide in the plasma 5 days after the onset of anuria are interpreted as manifestations of sulfonamide intoxication. Proteinuria persisting for a period of nearly 2 years can be attributed either to sulfonamide intoxication or to some other concurrent process, *e.g.* diffuse glomerulonephritis. The possibility that acute diffuse glomerulonephritis was superimposed cannot be excluded; but it does not change the significance of the pattern of acute renal functional impairment, which we believe is related to the prolonged period of anuria, rather than to a specific noxious agent. The total dosage of sulfathiazole is relatively small, but it is known that sulfonamide intoxication does not bear a uniform quantitative relationship to the amount of drug administered. Death from renal insufficiency has been reported in a 1-year-old child following the administration of 0.65 gram of sulfathiazole (4).

Smith has emphasized the limitations of the clearance methods in the diseased kidney (5). The use of mannitol as a measure of glomerular filtration is based on the assumption that none of the filtered mannitol is reabsorbed by the tubules. The markedly decreased mannitol clearance first observed following anuria cannot be taken as an accurate measurement of glomerular filtration, since the reduction may have been caused in part by tubular back-diffusion. However, we believe that the mannitol clearance is a reliable index of glomerular filtration 4½ weeks after the onset of anuria, since back-diffusion had apparently disappeared, as indicated by the positive value for Tm_{PAH} and the normal value for the blood non-protein nitrogen. Had tubular damage been extensive enough to permit back-diffusion of mannitol at this time, it is logical to assume that the more diffusible urea would also have diffused back, causing an elevation of the blood non-protein nitrogen. The anuria and azotemia during the acute episode may be attributed in part to glomerular and in part to tubular damage.

It is probable that the PAH extraction ratio was reduced in the first 2 series of observations. A decrease in this extraction ratio has been reported after extreme reduction of renal blood flow in the dog (4, 6 to 8). Hence, until the last observation, the PAH clearance cannot be taken as accurately indicating the renal blood flow. Although the normal value of the filtration fraction at 4½ weeks implies a normal extraction ratio at this time, tubular impairment was still present at 7 weeks, as manifested by the reduced value of Tm_{PAH}.

The negative Tm_{PAH} value after anuria indicates that PAH was diffusing back through the tubules at some point.[2] It may be suggested as a mere speculation that the tubular segment most susceptible to anoxic injury is the thin limb of the loop of Henle; on the supposition that PAH is excreted by the proximal tubule, a negative value of Tm_{PAH} does therefore not necessarily imply that this process of excretion was specifically impaired. though some impairment is certainly to be anticipated after a prolonged period of glomerular damage or vascular spasm. Such marked functional disorganization is probably indicative of wide-

[2] The maximal urine mannitol blanks observed in patients in uremia have been approximately 3.76 mgm. per minute (9). Such a correction applied to those determinations in this patient in which negative T_m values were observed would not result in positive values for T_m. However, in view of the low mannitol clearance, it seems probable that some back-diffusion of both mannitol and PAH occurs.

spread changes involving glomeruli, tubules, and interstitium, such as have been described at necropsy in patients with fatal sulfonamide intoxication (4). Luetscher and Blackman (10) reported an unusual increase in the concentration of serum sodium and chloride in patients with sulfathiazole intoxication at a time when oliguria and nitrogen retention were diminishing, and they correlated this finding with the renal lesions observed in the tubules at necropsy. At 3½ weeks, despite the fact that glomerular function had apparently returned to the normal range, the persistent negative value for $T_{m_{PAH}}$ indicates that the tubular cells were still so disorganized as to permit abnormal back-diffusion. It was not until 4½ weeks after the onset of anuria that a positive value for $T_{m_{PAH}}$ was obtained. The eventual return of $T_{m_{PAH}}$ to normal indicates that the tubular injury in this patient did not result in permanent loss of excretory capacity. Bradley (11) and Rubin (7) have observed negative values for $T_{m_{PAH}}$ in patients with diffuse glomerulonephritis.

The primary disturbance producing immediate and subsequent renal injury during anuria, whether the anuria is associated with diffuse glomerulonephritis, post-transfusion reaction, sulfathiazole intoxication, crush syndrome, battle casualties with peripheral circulatory failure, or ureteral trauma, may be renal ischemia. Theoretically, the course of events in this patient may have started with marked renal ischemia initiated reflexly from the ureter or from intrarenal pathology.

SUMMARY

Marked reduction in renal function was observed early in the postanuric stage in a patient with sulfathiazole intoxication.

The filtration rate returned fairly rapidly to normal. Tubular excretory capacity, however, was impaired for a longer period of time, but eventually returned to normal.

A negative value for $T_{m_{PAH}}$ indicating abnormal tubular back-diffusion of PAH persisted for approximately 4 weeks from the onset of anuria.

BIBLIOGRAPHY

1. Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease, Commonwealth Fund, New York, 1944, p. 212.
2. Barker, H. G., and Clark, J. K., Effect of p-aminohippurate on mannitol determinations by the periodate-iodide-thiosulfate method. Proc. Soc. Exper. Biol. and Med., 1947, 64, 120.
3. Chasis, H., Redish, J., Goldring, W., Ranges, H. A., and Smith, H. W., The use of sodium p-aminohippurate for the functional evaluation of the human kidney. J. Clin. Invest., 1945, 24, 583.
4. Murphy, F. D., Kuzma, J. F., Polley, T. Z., and Grill, J., Clinicopathologic studies of renal damage due to sulfonamide compounds. Arch. Int. Med., 1944, 73, 433.
5. Smith, Homer W., Note on the interpretation of clearance methods in the diseased kidney. J. Clin. Invest., 1941, 20, 631.
6. Corcoran, A. C., and Page, I. H., Effects of hypotension due to hemorrhage and of blood transfusion on renal function in dogs. J. Exper. Med., 1943, 78, 205.
7. Rubin, Mitchell I., Personal communication.
8. Selkurt, E. E., The changes in renal clearance following complete ischemia of the kidney. Am. J. Physiol., 1945, 144, 395.
9. Earle, David, Personal communication.
10. Luetscher, J. A., Jr., and Blackman, S. S., Jr., Severe injury to kidneys and brain following sulfathiazole administration; high serum sodium and chlorides levels and persistent cerebral damage. Ann. Int. Med., 1943, 18, 741.
11. Bradley, Stanley, Personal communication.

THE EFFECT OF CYTOCHROME C UPON THE RESPIRATION OF TISSUE SLICES

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Recent studies of the effect of added Cytochrome C upon the respiration of tissue homogenates (1, 2) have led to the conclusion that the organ content of this respiratory enzyme is less than optimum (2, 3). This has led to intensive investigation of the results of its parenteral administration to normal and anoxic animals and its effectiveness in clinical conditions characterized by tissue anoxia (2 to 8). In view of the theoretical and practical implications of these observations, the effect of Cytochrome C upon the respiration of normal and anoxic tissue slices has been investigated.

EXPERIMENTAL

Wistar strain rats, varying in weight from 100 to 400 grams, were used throughout this study. They were fed Purina Dog Chow ad lib, and were not fasted before sacrifice. In all experiments the rats were killed by a sharp blow on the head. The heart or liver was excised as rapidly as possible and placed in an iced, phosphate-buffered medium which contained 0.2 per cent glucose and whose ionic composition was as follows:

Cation	Concentration	Anion	Concentration
	meq. per l.		*meq. per l.*
Na	150	Cl	142
K	4	HPO_4 }	15
Ca	2	H_2PO_4 }	
Mg	1		

The pH was 7.4.

Liver slices approximately 0.5 mm. in thickness were prepared by means of a modified Stadie and Riggs tissue microtome (9). Heart slices of similar thickness were made by hand, using a razor blade as cutting tool and a glass slide as a template. Both types of slices were placed in an aliquot of well-oxygenated incubation medium at room temperature until placed in Warburg vessels.

Respiration was measured at 37° C. by the direct method of Warburg. Each vessel contained 3.0 ml. of the incubation medium in the outer compartment and 0.2 ml. of 10 per cent KOH plus filter paper in the center cup. Approximately 20 mgm. of tissue (dry weight) were used in each vessel. The vessels were equilibrated with varying mixtures of oxygen and nitrogen while being shaken in the constant temperature bath at the rate of 120 oscillations per minute. Readings were started about 30 minutes after the animals had been sacrificed. The tissue was removed at the end of the experiment and the dry weight obtained. All determinations were made in duplicate or triplicate. The results for the liver slices usually checked within 10 per cent and for the heart slices within 15 per cent. Respiration rate curves were plotted and the best linear relationship obtained from the observed points. Results were expressed as QO_2 ($mm.^3$ of O_2 per mgm. of dry weight of tissue per hour).

Cytochrome C was prepared according to the method of Keilin and Hartree (10). The preparation was carried only as far as the stage known to yield a product containing .34 per cent iron. The purity of the material was based on its spectrophotometric analysis using a Beckmann spectrophotometer with 1 cm. cells. The optical density of a solution containing a known amount of the preparation was determined at a wave length of 550 mμ and a spectral interval of 1.5 mμ. The concentration was then calculated using the absorption constant proposed by Drabkin (11). The preparation used consisted of 55 per cent Cytochrome C and 45 per cent sodium chloride. Solutions were freshly prepared from the lyophilized powder on the day of each experiment. The amount of Cytochrome C added to the suspending medium was equal to or in excess of the normal concentration in the tissue studied (12).

RESULTS

The results of respiration measurements on liver slices at varying oxygen tensions with and without added Cytochrome C are summarized in Table I. Respiration was measured over a period of 1 hour in slices equilibrated with 2.5 to 20 per cent oxygen, following which 100 per cent oxygen was introduced and the respiration during recovery measured for a second hour. In addition, liver slices were equilibrated with commercial nitrogen (ca. 0.5 per cent oxygen) for a period of 40 minutes following which 100 per cent oxygen was introduced and the rate of respiration measured for 1 hour. During the period of equilibration with nitrogen, there was no measurable respiration. It is apparent from the data presented

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TABLE I

Rat liver

Experiment number	Cytochrome C	QO_2 without Cyt. C	QO_2 with Cyt. C	QO_2 during anoxia without Cyt. C	QO_2 during anoxia with Cyt. C	QO_2 on recovery without Cyt. C	QO_2 on recovery with Cyt. C
	gamma per ml.	100 per cent O_2					
1	300	7.6	7.2				
2	300	5.9	5.9				
3	1200	7.0	6.5				
		Ave. 6.8	6.5				
		100 per cent O_2		20 per cent O_2		100 per cent O_2	
4	300	6.0		4.3	3.1	5.8	4.6
5	500	8.7		5.0	4.7	7.4	6.5
6	500	9.2		5.2	5.6	7.4	7.0
7	500	8.1		4.6	5.0	7.4	7.6
		Ave. 8.0		4.8	4.6	7.0	6.4
		100 per cent O_2		5 per cent O_2		100 per cent O_2	
8	500	9.0		1.9	2.4	6.1	6.1
9	500	8.3		2.4	2.5	7.0	7.0
10	500	8.5		2.0	2.0	7.3	6.8
		Ave. 8.6		2.2	2.3	6.8	6.6
		100 per cent O_2		2.5 per cent O_2		100 per cent O_2	
11	500	9.5		1.8	1.6	7.7	7.2
12	500	8.9		1.2	1.3	5.8	6.6
13	500	7.1		1.4	1.6	5.8	6.0
		Ave. 8.5		1.5	1.5	6.4	6.6
		100 per cent O_2		0.5 per cent O_2		100 per cent O_2	
14	500	7.7				6.0	5.6
15	500	9.6				7.6	6.6
16	500	9.2				7.2	7.4
		Ave. 8.8				6.9	6.5

that the addition of Cytochrome C had no demonstrable effect upon the rate of respiration of surviving liver slices. The metabolism of those slices which were exposed to oxygen concentrations below 100 per cent was reduced in all cases. The magnitude of the depression was roughly proportional to the oxygen tension. Moreover, restoration of such anoxic slices to an atmosphere of pure oxygen resulted in a marked increase in the QO_2. All returned to a level of approximately 6.5. However, the presence of Cytochrome C in concentration of 500 gamma per ml. did not cause an increase in oxygen consumption either during the period of exposure to low oxygen tension or after the tissue was returned to an atmosphere of pure oxygen. The oxygen consumption of liver tissue subjected to varying degrees of anoxia was in no way affected by the presence of greater than physiological

TABLE II

Rat heart

Experiment number	Cytochrome C	QO_2 without Cyt. C	QO_2 with Cyt. C	QO_2 during anoxia without Cyt. C	QO_2 during anoxia with Cyt. C	QO_2 on recovery without Cyt. C	QO_2 on recovery with Cyt. C
	gamma per ml.	100 per cent O_2					
1	500	7.7	6.1				
2	300	9.0	7.1				
	900		8.2				
3	500	11.1	9.6				
4	500	12.7	13.2				
		Ave. 10.1	8.8				
		100 per cent O_2		20 per cent O_2		100 per cent O_2	
5	500	10.7		3.6	3.3	4.5	4.6
6	500	12.3		4.9	4.5	4.8	6.8
7	500	12.0		4.3	4.2	5.7	5.4
8	500	11.5		4.3	3.4	6.6	5.6
		Ave. 11.6		4.3	3.9	5.4	5.6
		100 per cent O_2		10 per cent O_2		100 per cent O_2	
9	500	7.1		1.8	1.4	3.5	3.6
10	500	9.8		2.0	2.0	3.3	3.6
11	500	6.4		2.0	2.1	3.9	3.7
12	500	7.7		2.5	2.4	2.5	3.6
		Ave. 7.8		2.1	2.0	3.3	3.6
		100 per cent O_2		5 per cent O_2		100 per cent O_2	
13	500	8.6		0.8	0.7	2.9	2.0
		100 per cent O_2		0.5 per cent O_2		100 per cent O_2	
14	500	11.1				3.7*	3.4*
15	500	12.7				3.7*	3.9*
16	500	11.5				4.3	4.3
17	500	6.9				2.7	3.2
		Ave. 10.6				3.6	3.7

* Single determination.

concentrations of Cytochrome C in the suspending medium.

Table II comprises a summary of essentially similar data resulting from studies of heart slices. It is apparent that the metabolism of heart slices was depressed by anoxia to a greater extent than in the case of liver slices exposed to the same oxygen tension. However, they did not show the same degree of recovery when returned to an atmosphere of 100 per cent oxygen. Cytochrome C had no effect upon the respiration of heart slices under any of the conditions considered.

CONCLUSIONS

1. The addition of Cytochrome C to the incubation medium did not alter the rate of oxygen consumption of surviving liver or heart slices equilibrated with 100 per cent oxygen.

2. The reduction of respiration of heart and liver slices induced by lowering the oxygen tension was not affected by the addition of Cytochrome C to the suspending medium.

3. The presence of Cytochrome C in the incubation medium of anoxic tissue slices did not affect their respiration after restoration to an atmosphere of 100 per cent oxygen.

4. The failure of added Cytochrome C to affect the rate of respiration of surviving tissue slices may have been due to non-penetration of Cytochrome C into the tissue cells or, if it penetrated, the increased concentration of Cytochrome C did not stimulate the rate of oxygen consumption.

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BIBLIOGRAPHY

1. Schneider, W. C., and Potter, V. R., The assay of animal tissues for respiratory enzymes; succinic dehydrogenase and cytochrome oxidase. J. Biol. Chem., 1943, 149, 217.
2. Proger, S., Dekaneas, D. J., and Schmidt, G., Some observations of the effect of injected Cytochrome C in animals. J. Clin. Invest., 1945, 24, 864.
3. Proger, S., Dekaneas, D., and Schmidt, G., The increase of Cytochrome C content of organs following its parenteral injection. J. Clin. Invest., 1944, 23, 949.
4. Proger, S., Dekaneas, D., and Schmidt, G., The effects of anoxia and of injected Cytochrome C on the content of easily hydrolyzable phosphorus in rat organs. J. Biol. Chem., 1945, 160, 233.
5. Proger, S., Some effects of injected Cytochrome C in animals and man. Bull. New England M. Center, 1945, 7, 1.
6. Proger, S., and Dekaneas, D., Some further observations on parenteral use of Cytochrome C with special reference to cerebral anoxia and shock. Bull. New England M. Center, 1945, 7, 149.
7. Proger, S., and Dekaneas, D., The use of Cytochrome C in combating tissue anoxia. Science, 1946, 104, 389.
8. Proger, S., and Dekaneas, D., Some effects of injected Cytochrome C in myocardial and cerebral anoxia in man. J. of Ped., 1946, 29, 729.
9. Stadie, W. C., and Riggs, B. C., Microtome for the preparation of tissue slices for metabolic studies of surviving tissues in vitro. J. Biol. Chem., 1944, 154, 687.
10. Keilin, D., and Hartree, E. F., Purification and properties of Cytochrome C. Biochem. J., 1945, 39, 289.
11. Drabkin, D. L., Spectrophotometric studies. J. Biol. Chem., 1941, 140, 373.
12. Crandall, M. W., and Drabkin, D. L., Cytochrome C in regenerating rat liver and its relation to other pigments. J. Biol. Chem., 1946, 166, 653.

THE EFFECT OF FATAL *P. KNOWLESI* MALARIA ON SIMIAN CIRCULATORY AND BODY FLUID COMPARTMENT PHYSIOLOGY [1]

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Although some objective measurements of the altered physiology of the malarial host have been made in human patients with therapeutic *P. vivax* and *P. falciparum* infections (1), almost no such studies have been made of the circulatory and fluid compartment volumes in fatal malaria. Numerous conclusions regarding the altered fluid physiology of the monkey with fatal *P. knowlesi* infections have been made by Knisely, *et al* (2), but these were, essentially, inferences based on microscopic observations of the capillaries (omentum, stomach, intestine and peritoneum) of living animals. The present investigation was made to elicit objective information concerning the altered physiological mechanisms in monkeys fatally ill of malaria.

MATERIALS AND METHODS

The following control determinations were made in 23 normal *Macacus mulata* monkeys who varied in weight from 2.45 to 4.40 kgm.: (*a*) total plasma volume by a modification (3) of the T-1824 dye dilution method of Gibson and Evans (4), (*b*) NaSCN ("extracellular") volume by a modification (3) of the method of Crandall and Anderson (5), (*c*) plasma protein concentration by the $CuSO_4$ method of Phillips, Van Slyke, *et al* (6), (*d*) arterial and venous blood oxygen content by the microgasometric procedure of Roughton and Scholander (7), (*e*) circulation times by a modification (3) of the fluorescein method of Fishback, *et al* (8), and (*f*) venous hematocrit values using Wintrobe tubes and centrifuging the heparinized blood for ½ hour at 3,000 r.p.m.

The total blood volume (TBV) was calculated from the measured plasma volume and average venous hematocrit value. The circulating red cell mass (TRBC) was calculated by subtracting the plasma volume from the total blood volume. The total circulating protein (TCPr.) was derived from the plasma protein concentration and the plasma volume. Each fluid volume measurement was related to the body weight of the animal at the time of the determination.

Immediately following the control determinations, 16 of the animals were infected with *P. knowlesi* by intravenous injection of small amounts (0.25 to 2.00 ml.) of whole blood from other animals with acute infections. The survival period of these experimental animals was 4 to 12 days with an average of 7.1 days. A total of 32 sets of the above determinations were carried out on the 16 animals, beginning on the second day post-inoculation and extending up to within ½ hour of death. Determinations of the level of parasitemia were made at least daily on each animal.

Following the withdrawal of the last dye-NaSCN sample, 1 ml. of femoral arterial and 1 ml. of femoral venous blood were airlessly withdrawn as near simultaneously as possible (within 2 minutes) and the oxygen content *immediately* determined by the Roughton-Scholander method. Immediate measurement of the O_2 content is imperative in the blood of the malarious animal (especially those at high parasitemic levels) because the parasites use oxygen at a rapid rate. Allowing the samples to stand, even using the precaution of storage at low temperatures as described by Roughton (7) for normal blood, will lead to gross errors in the true O_2 content of paludic blood.

At the end of each set of determinations, *i.e.*, after the O_2 content of arterial and venous blood and the circulation time had been measured, the femoral triangle was closed by 3 or 4 skin clips and the animal returned to the cage. Since no sterile precautions were observed on exposing the artery and vein, some of the animals were given 10,000 units of penicillin intramuscularly to minimize local infection. However, such precaution was not always taken and the few skin-clip infections which developed were treated as soon as they were discovered. The right and left femoral triangles were alternated for serial studies. Local anesthesia was employed by infiltration of the femoral triangle region with 2 ml. of 2 per cent procaine.

More pronounced alterations in fluid and circulatory physiology were anticipated as the parasitemic level rose. Multiple measurements at each parasitemic level were desirable, but not always feasible. Consequently, the results have been analyzed by grouping the data in a logical but arbitrary manner. It was noted that if less than 10 per cent of the red cells of the monkey were parasitized, the animal, while showing definite clinical signs (increased body temperature, reduced hematocrit, increased "extra-

[1] The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Tennessee.

[2] Lieutenant-Colonel, M.C., AUS.

cellular" fluid volume), is not acutely ill. At such levels the fatal course may be readily averted by adequate chemotherapy. Likewise, such animals are alert and active; peripheral blood can be readily drawn; and the monkey has the outward appearance of a normal animal.

However, with parasite levels at which more than 10 per cent of the red cells are parasitized, the fatal course is not easily reversed by chemotherapeutic intervention; the animals may be myasthenic and often appear depressed; it becomes increasingly difficult to draw blood from the peripheral veins or, at times, even to obtain capillary blood for smears. These conditions continue and become progressively more pronounced until death. Thus, we have divided the determinations made in this series into 4 groups: (*1*) normal (control) values, (*2*) values obtained on animals having less than 10 per cent parasitemia, (*3*) values obtained on animals having greater than 10 per cent parasitemia, and (*4*) values obtained within the 24-hour period preceding death.

RESULTS

In general the data are presented and discussed by comparing the averages of the 4 groups (Tables I, II). Entirely similar results were obtained by calculating the changes in individual animals.

Plasma volume. Figure 1 shows the changes in plasma volume expressed as ml. per kgm. of body weight. It will be seen that the plasma volume increases throughout the course of the disease until just before death. Red cell destruction is a constant feature of active malaria; and, therefore, an increase in plasma volume is not unexpected, since the usual physiological mechanism for maintaining a constant blood volume in the presence of erythrocyte loss is plasma dilution. The terminal fall in plasma volume may reflect either (*1*) pooling of blood in regions which the dye cannot penetrate except by diffusion or (*2*) increased capillary leakage. However, none of the plasma volume alterations reveal statistical significance (Table II).

Blood volume. Although the plasma volume shows progressive enlargement until just before death from malaria, the whole blood volume is reduced early in the disease. It is obvious that

TABLE I

*Comparison of average values in groups 1, 2, 3 and 4**

Group	Hct.†	TPV	PV per kgm.	TBV	BV per kgm.	TEFV	EFV per kgm.	TRBC	RBC per kgm.	Pl. Pr.	TCPr.	A-V O_2	C. T.
1	40.4	163.3	44.9	273.1	75.1	725.8	208.9	110.2	29.9	6.97	11.3	6.9	9.5
s.d.	5.2	52.9	8.9	83.5	13.4	169.5	43.4	39.2	6.4	0.72	4.2	2.2	2.2
s.e.	1.1	11.3	1.9	17.8	2.9	43.8	11.2	2.7	1.4	0.17	0.3	0.4	0.7
2	31.1	158.8	46.4	231.5	67.9	1046.0	322.4	72.7	21.3	6.94	10.6	6.4	9.3
s.d.	8.1	32.9	8.4	47.6	12.4	473.6	130.7	27.1	8.3	0.64	2.1	2.5	2.4
s.e.	2.0	8.2	2.1	11.9	3.1	131.2	36.3	6.8	2.1	0.17	0.6	0.9	0.7
3	24.2	185.2	51.6	246.2	68.1	1071.9	314.1	61.1	16.5	6.80	12.7	6.2	9.0
s.d.	6.7	26.0	6.4	41.1	7.0	398.9	111.5	23.6	5.0	0.74	1.4	2.1	2.9
s.e.	2.6	9.8	2.4	15.6	2.7	178.4	49.9	8.9	1.9	0.32	0.6	1.5	2.0
4	22.4	133.6	38.0	157.2	48.5	1077.9	344.8	33.6	7.8	6.44	7.9	7.7	19.5
s.d.	10.6	43.2	12.7	40.4	11.3	357.0	82.8	10.4	3.7	0.36	2.5	2.3	10.5
s.e.	4.3	19.3	5.7	18.1	5.1	145.7	33.8	4.7	1.7	0.41	1.1	1.3	5.2

* Group 1—normal values (23 monkeys).
Group 2—animals with less than 10 per cent parasitemia (17 animals).
Group 3—animals with greater than 10 per cent parasitemia at least 30 hours before death (8 animals).
Group 4—animals measured on the day of death (all had greater than 10 per cent parasitemia) (7 animals).

† Hct.—hematocrit in per cent.
TPV—total plasma volume in ml.
PV per kgm.—plasma volume in ml. per kgm.
TBV—total blood volume in ml.
BV per kgm.—blood volume in ml. per kgm.
TEFV—"extracellular" fluid volume in ml.
EFV per kgm.—"extracellular" fluid volume in ml. per kgm.
TRBC—total erythrocyte mass in ml.
RBC per kgm.—erythrocyte mass in ml. per kgm.
Pl. Pr.—plasma protein concentration in grams per cent.
TCPr.—total circulating protein in grams.
A–V O_2—arterio-venous oxygen difference in volumes per cent.
C. T.—circulation time (femoral vein to tongue) in seconds.

TABLE II

Statistical comparison (T values) of circulatory and fluid compartment physiology in groups 1, 2, 3, and 4†

Between groups	Hct.	TPV	PV per kgm.	TBV	BV per kgm.	TEFV	EFV per kgm.	TRBC	RBC per kgm.	Pl. Pr.	TCPr.	A-V O_2	C. T.
1 and 2	4.04*	0.32	0.57	1.94	1.71	2.31	2.99*	5.16*	3.28*	0.01	0.06	0.45	0.20
1 and 3	5.85*	1.46	2.18	1.14	1.80	1.88	2.06	5.29*	5.73*	0.47	1.25	0.47	0.24
1 and 4	3.96*	1.33	1.16	4.57*	4.59*	2.31	2.82*	14.29*	10.18*	2.36	2.28	0.54	1.89
3 and 4	0.36	2.68	2.21	3.73*	3.43*	0.03	0.51	2.75*	3.44*	1.04	3.75*	0.74	1.87

* These values are significant at the 1 per cent level.
† Footnotes in Table I applicable also to Table II.

this reduction is entirely due to red cell destruction which begins with, or shortly after, inoculation. However, the reduction in whole blood volume is not, in itself, serious until the terminal stages are reached when the average reduction is 42.4 per cent. It is highly probable that such a loss may lead to circulatory failure and a fatal outcome.

Erthrocyte mass. The loss in circulating red cell volume throughout the course of fatal *P. knowlesi* infections is almost linear (Figure 1). The average terminal reduction reached the high figure of 73.9 per cent at which time the average red cell mass per kgm. was but 7.8 ml. Neither the whole blood volume nor the hematocrit (Figure 1, Table I) shows parallel alterations due to the fact that the plasma volume increases in early malaria and does not fall as precipitously as does the red cell volume in the terminal stages.

Plasma protein. While the plasma protein concentration shows a small but progressive reduction during the course of the disease (Figure 2, Table I), the total circulating protein increases until just before death. The explanation for these results lies in the observed fact that there is a gradual increase in plasma volume until the day of death. The terminal fall in total circulating protein (which is statistically significant [Table II]) indicates again that increased capillary leakage may occur near the end of the fatal course.

Arterio-venous oxygen difference. The difference between the O_2 content of arterial and venous blood reveals minor changes during early malaria (Figure 2, Table I) with a marked terminal rise. Due to the drastically reduced blood volume and prolonged circulation time at this stage, the volume flow throughout any portion of the capillary bed must be materially reduced terminally.

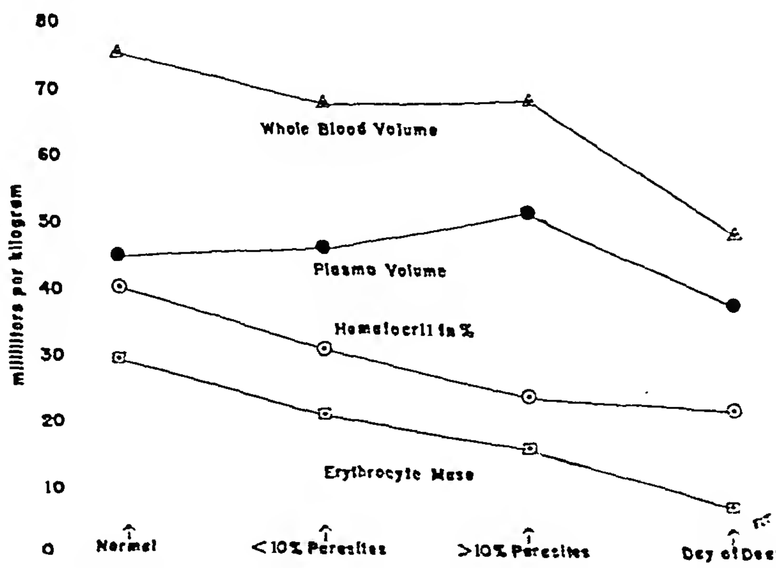

FIG. 1. CHANGES IN BLOOD AND PLASMA VOLUMES IN FATAL SIMIAN MALARIA

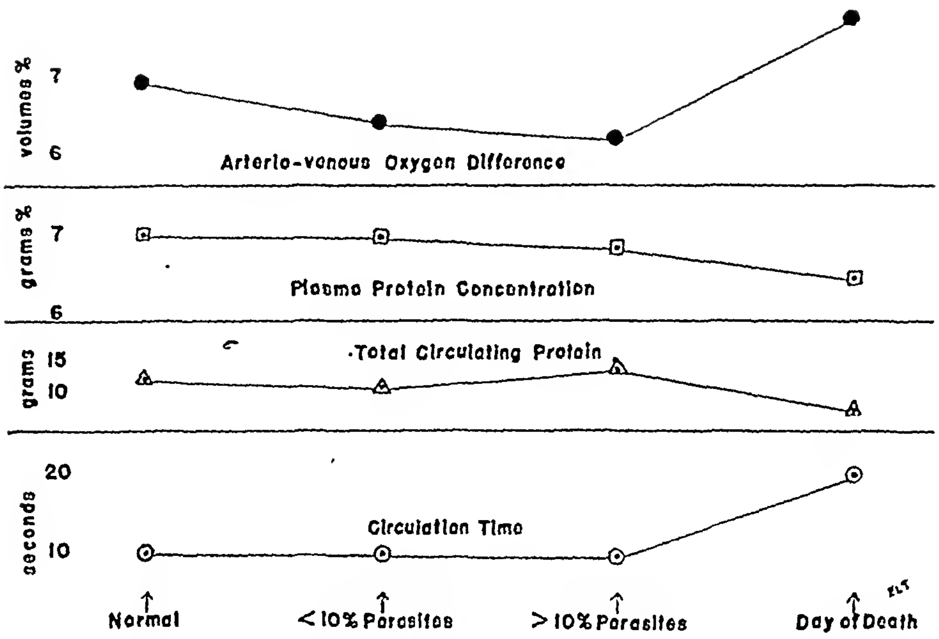

FIG. 2. AVERAGE PHYSIOLOGICAL ALTERATIONS IN FATAL SIMIAN MALARIA

Therefore, such an increase in A-V difference at this time is not unexpected.

Circulation time. During the early stages of malaria, the circulation time shows minor reductions (Figure 2, Table I). That this is a concomitant of dilution of the blood (loss of red cells and increase of plasma) and a lowered viscosity is highly probable. Terminally there may be a doubling of the circulation time. Our results indicate that although such a terminal slowing of the circulation could be due to intravascular agglutination which apparently occurs at this time (2), it could likewise be ascribed to vasoconstriction which occurs to compensate for the reduced circulatory volume.

"Extracellular" fluid volume. The volume of fluid which is available for the dilution of NaSCN ("extracellular") increases in a progressive manner throughout the fatal course of *P. knowlesi* infections in the monkey. Actually, the grouped data (Figure 3, Table I) mask some of the more startling alterations of this volume in some of the animals. Usually the *apparent* "extracellular" volume exceeded 60 per cent of the body weight

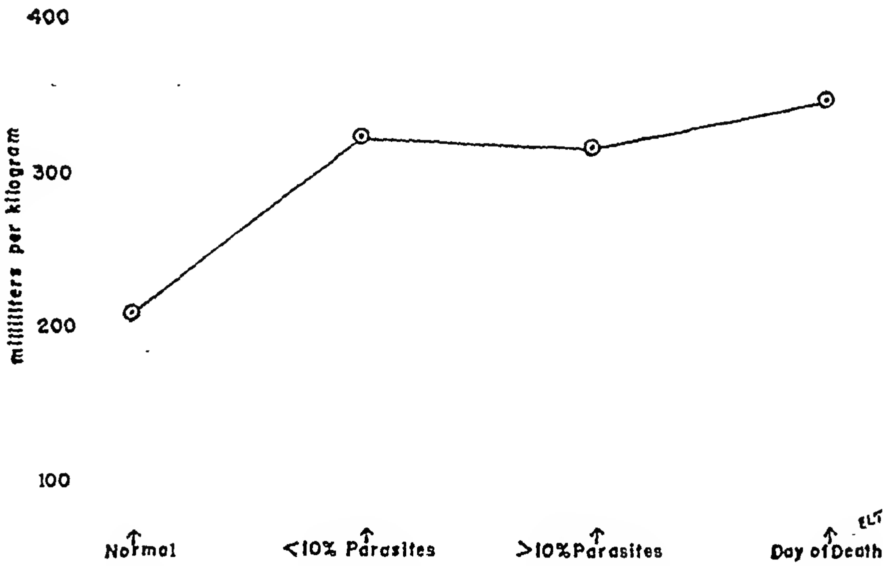

FIG. 3. "EXTRACELLULAR" FLUID VOLUME IN FATAL SIMIAN MALARIA

just preceding death of animals whose survival period and consequently the febrile period was prolonged (10 to 12 days).

DISCUSSION

Feldman and Murphy (1), who measured the blood and plasma volumes in sporozoite-induced malaria in the human being, pointed out that "the plasma volume is increased almost without exception during the active phase of *vivax* and *falciparum* infections studied." On the other hand, Knisely, *et al* (2) inferred from microscopic observations of the capillaries of living monkeys infected with *knowlesi* that "there is a progressively decreased circulating plasma volume."

From the measurements reported here, it is now possible to resolve these 2 divergent views. In early malaria (in fact, up until the day of death in simian malaria), the plasma volume is *progressively* increased. The patients studied by Feldman and Murphy, while seriously infected, were not usually in the final stages of the disease. Indeed, there were noted in 1 patient (described as being in shock) plasma and blood volume reductions of 46 and 57 per cent, respectively. This patient recovered following transfusion with whole blood and plasma. Knisely, *et al*, on the other hand, were observing the alterations in the monkey infected with an almost uniformly fatal type of malaria, and their conclusions were based primarily on observations made near the time of death.

As was observed in the human being (1), the monkey shows a marked and progressive fall in the venous hematocrit value. These observations do not support the subjective inference of Knisely that at times "the venous blood shows high red cell counts (hemoconcentration)."

Since the early reduction in circulating blood volume is due entirely to red cell destruction, it follows that the oxygen-carrying capacity of the blood will be reduced by at least a similar percentage. Indeed, since the presence of the parasite within the red cell reduces its ability to carry oxygen (by destroying hemoglobin) (9), one would expect an even greater decrease in the O_2 capacity of the arterial blood than the anemia would indicate.

The severe reduction in the number of circulating red cells and in the ability of some of the circulating cells to carry oxygen is associated, at least terminally, with a *100 per cent* increase (prolongation) of the circulation time. That tissue anoxia will result is an obvious corollary of these findings.

At the outset, the progressive *apparent* increase in the size of the "extracellular" compartment in simian malaria was difficult to explain. As previously mentioned, animals having the longest periods of survival (10 to 12 days) revealed *apparent* "extracellular" volumes which exceeded 60 per cent of the body weight. Since the plasma volume and the body weight fall terminally, it is evident that in the malarious monkey NaSCN no longer measures the "extracellular" water. One of the present authors (10) has shown that in febrile conditions SCN enters the tissue cells and becomes diluted by the entire water mass of the body.

Since the tissue cell, at elevated body temperatures, becomes permeable to the SCN ion which it normally excludes, it follows that cellular permeability to native ions might also be altered under the same or similar conditions. Pinelli (11), Velick and Scudder (12), Zwemer, Sims and Coggeshall (13), Flosi (14), and Morin, Bader, Monnier and Moreau (15) have all reported a decrease in blood sodium or chloride and/or an increase in blood potassium concentration in malaria.

Our observations indicate that such changes may occur as a result of altered cellular permeability in febrile states. That the entrance of Na and Cl into cells and the loss of K from cells might be accompanied by severe metabolic disturbances leading to a fatal outcome cannot be overlooked.

SUMMARY AND CONCLUSIONS

1. Repeated measurements of plasma, blood and "extracellular" fluid volumes, hematocrit values, plasma protein concentration, arteriovenous oxygen differences, and circulation time have been made in 16 *Macacus mulata* monkeys with fatal *P. knowlesi* infections.

2. Statistically significant alterations in some of these values appear early in the disease and

by mosquito inoculation without the development of clinical jaundice.[2]

MATERIALS AND METHODS

The patients in the blood inoculation group were given initially either the McCoy strain of *P. vivax* or the McLendon strain of *P. falciparum*. After 3 to 6 days of fever they received an antimalarial drug under study. If there was complete suppression of parasites for 2 or 3 weeks, they were then reinoculated from another donor and the second course of fever was allowed to run to a spontaneous termination. If this did not give them the required 75 hours of fever over 103°, they were inoculated with *P. malariae* or given a course of triple typhoid vaccine infusions. Thus most patients were inoculated more than once and their stay on the wards ranged from 1 to 6 months. Inoculations were performed by transferring from 0.1 to 4.0 cc. of citrated blood directly from one patient to the other, the amount depending on the parasite density of the donor's blood.

Some characteristics of the patients treated are important in evaluating the results. They were almost all referred by the Social Hygiene Clinics of the New York City Board of Health. In general they were from an economic stratum that is more prone to dietary deficiencies and alcoholism than the average civilian or soldier with naturally occurring malaria. In addition about 50 per cent had received fairly extensive arsenical treatment; only 11 per cent had received no prior antiluetic treatment.

The bromsulfalein excretion was examined routinely on admission so that observations are available on all but a few patients. The 5 mgm. per kgm. dose was used. Any retention of over 15 per cent in 30 minutes, with a 5-minute specimen designated as 100 per cent, was considered abnormal. Of 400 patients on whom the test was done before treatment only 72 per cent were normal by these criteria. By contrast, all but 1 of the 27 conscientious objectors had readings well under 15 per cent, and the 1 exception had 2 determinations of 14 and 18.

RESULTS

The only significant difference in history and preliminary work-up between patients who later developed jaundice and those who did not was in bromsulfalein retention. As shown in Table I, the former group had a significantly higher incidence of liver impairment demonstrated by that test than 100 patients picked at random from among those who did not develop jaundice. Differences were observed in the amount of previous exposure to the arsphenamines and excessive indulgence in alcohol, but these are not statistically significant. The age and sex range of the jaundiced patients was the same as that of the group as a whole. There was a somewhat higher incidence in those of the white race, but some attacks of jaundice may well have been missed in the negroes. No strain of malaria was associated more often with jaundice than any other.

Half of the 36 patients became jaundiced while they were febrile with malaria. These will be referred to hereafter as Group 1. Those occurring in the post-febrile period will be designated as Group 2. Of the 18 patients in Group 1, 6 occurred during the first bout of fever, 11 during the second and 1 during the third. In Group 2, 4 occurred after the first bout, 12 after the second and 2 after the third.

Fifty per cent of the patients in Group 1 and 88 per cent of these in Group 2 had either mild symptoms or none at all. The remainder were more severely ill with such symptoms as marked anorexia, vomiting, diarrhea, epigastric pain, pruritis, and in 1 case hematemesis and ascites. None died. Almost all had enlargement of the liver. The average duration of jaundice in Group 1 was 13 days and in Group 2, 7 days. The diagnosis of hepatitis was confirmed by tests for urinary bile and urobilinogen, icteric index or serum bilirubin and bromsulfalein excretion. In Group 1, 7 patients had no drop in hemoglobin over the 7 to 10 day period prior to the onset of jaundice, and 11 had an average drop of 1.3 grams, no more than the average patient has with the same amount of malaria.

TABLE I

Incidence of abnormal bromsulfalein retention among patients prior to blood inoculation to establish malaria

21 patients who developed jaundice compared with 100 patients picked at random who did not develop jaundice.

	Per cent bromsulfalein retention in 30 minutes				
	<16%	<21%	<31%	<41%	<51%
Patients with jaundice, *number*	12	15	18	20	21
Patients with jaundice, *per cent*	57%	71%	86%	95%	100%
Patients without jaundice, *number* and *per cent*	82	90	99	100	

[2] The author wishes to thank Dr. David P. Earle and Dr. Gordon Zubrod for supplying the data on these patients.

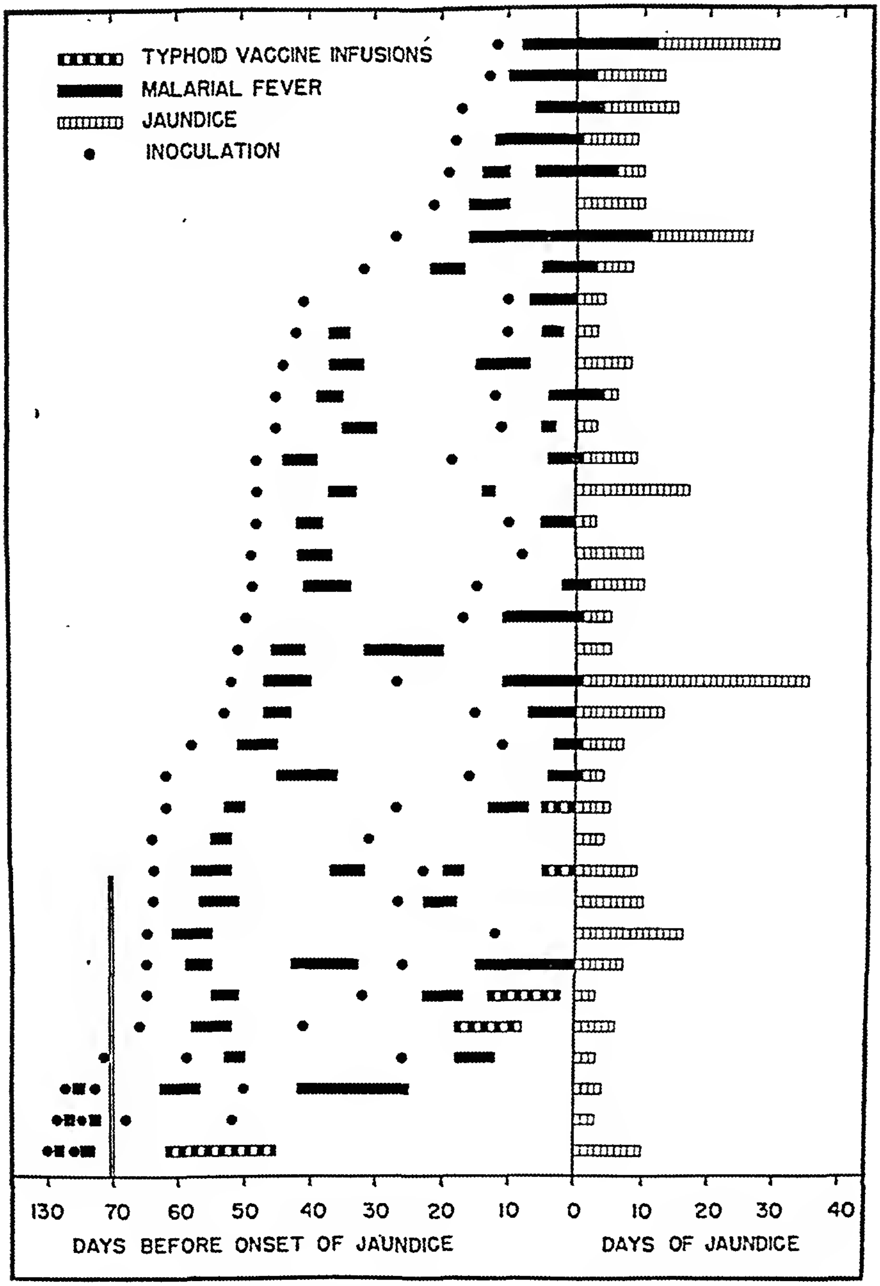

FIG. 1. SCHEMATIC REPRESENTATION OF THE TIME RELATIONSHIPS OF THE INOCULATIONS WITH BLOOD TO INDUCE MALARIA, THE DURATION OF MALARIAL AND TYPHOID VACCINE FEVER, AND THE ONSET AND DURATION OF JAUNDICE

The day of onset of the jaundice occurs at the zero line; the scale changes at the far left.

The relationship between the time of inoculation and the onset of jaundice is presented in Figure 1. These data are presented in detail to illustrate the possibility of a transfer of an icterogenic virus with the transfer of blood utilized for inoculation of patients with malaria. In Table II the figures are broken down into groups and reveal that those who developed jaundice during malaria had in general shorter "incubation periods" than those who developed their jaundice in a malaria-free

TABLE II

Number of days between inoculation and onset of jaundice (taking the maximum number of days when 2 or more inoculations were performed)

Group 1 includes those who developed jaundice while febrile with malaria, Group 2 those who developed it after their malaria had been terminated.

Days	Group 1	Group 2
0– 40	7	1
41– 55	8	6
56– 70	3	7
71–130	0	4

interval. In 28 of the patients, 78 per cent, the period between the first inoculation and onset of the jaundice was over 40 days. All patients were on the wards at least 3 weeks before the onset of jaundice.

There is additional data suggesting the possibility of transfer of an icterogenic agent in inoculation malaria. In 2 instances jaundice developed in each of 3 patients who had received their infected blood from a single donor, and there were 4 patients who donated blood to 2 others who later developed jaundice. The intervals in the former group were 12, 32 and 111 days in one instance, and 17, 26 and 42 days in the other. In the latter group the intervals were 8 and 16, 10 and 51, 44 and 118, and 53 and 66 days. The records available do not indicate how many patients received blood from these same donors and did not develop jaundice.

DISCUSSION

As far as is known, none of the drugs used in these studies to cure the malaria is hepatotoxic in the doses administered. In only 1 of the patients could hepatitis possibly be related to a drug, and that was a sulfonamide which was given to other patients who did not develop hepatitis. There is no reason to believe that the typhoid vaccine infusions caused the jaundice. Some of the cases may have been sporadic infectious hepatitis. There were always 15 to 30 patients in various stages of treatment associating closely with each other on the male ward. The cases were evenly distributed over a 2½-year period. One nurse developed typical acute hepatitis during this period without known contact outside the hospital.

Since no attempt was made to prove the transmission of a virus in any of the above reported patients, the evidence for such an etiology of some of the jaundice occurring during and after therapeutic malaria is only suggestive. But the fact that in half of the patients hepatitis occurred after the termination of malaria suggests that some of that occurring during malaria may be of the same etiology.

The incubation periods are on the whole considerably shorter than those reported for homologous serum jaundice. Incubation periods as short as 2 weeks, however, have been reported (9). Neefe *et al* (10) have reported changes in liver function tests in from 12 to 35 days after inoculation, although jaundice did not appear until considerably later. It is possible that the combination of an arsphenamine-injured liver and an intervening debilitating disease such as malaria may considerably shorten the time ordinarily required for the development of jaundice.

Of great importance with regard to this work are the recent reports by Neefe *et al* (11) and by Havens (12) that the parenteral transfer of a virus isolated from an epidemic of infectious hepatitis was followed by the same incubation period as the oral transfer, 20 to 37 days. Their data strongly suggest that there are at least 2 strains of hepatitis virus, and it is possible that strains with short as well as long incubation periods may have been responsible for our cases.

The occasional occurrence of jaundice, after arsphenamines have been resumed following malaria, may well be homologous serum jaundice rather than the toxic effect of arsphenamines on top of that of malaria as suggested by Kopp and Solomon (1). Indeed, there may be a distinct similarity between the problems of arsphenamine and malaria hepatitis in luetic patients. The former has recently been shown to be caused largely by transmission of a virus through improperly sterilized equipment (13, 14), a plausible explanation for the increased incidence of arsphenamine hepatitis during epidemics of infectious hepatitis.

Since the disease may be extremely serious, and since immune globulin has been shown to prevent infectious hepatitis if given early enough (15, 16), it might be wise to give immune globulin to each patient inoculated by transfer of infected blood. The evidence for its effectiveness in preventing post-transfusion hepatitis is not as strik-

ing, but it was successful in a study reported by Grossman *et al* (17).

The data presented suggest that care should be exercised in drawing conclusions about the effect of naturally occurring malaria on the liver from studies of blood inoculation malaria in syphilitic patients. The returned troops with relapsing malaria have, on the whole, more normal livers to start with, they are inoculated by mosquitoes rather than the transfer of human blood, and their malaria is usually treated promptly rather than allowed to run for 1 to 2 weeks.

SUMMARY

Thirty-six instances of jaundice complicated the treatment of 450 syphilitics with malarial fever induced by blood inoculation. Only half of these occurred during an actual attack of malaria.

None of 99 mosquito-inoculated patients developed jaundice.

The possibility that some of these attacks of hepatitis were caused by a specific icterogenic virus is discussed.

Evidence is presented to show that an appreciable number of patients with central nervous system syphilis have impaired hepatic function before they are given malaria.

ACKNOWLEDGMENT

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BIBLIOGRAPHY

1. Kopp, I., and Solomon, H., Liver function in therapeutic malaria. Am. J. M. Sc., 1943, 205, 90.
2. Fredericks, M. G., and Hoffbauer, F. W., A study of hepatic function in therapeutic malaria. J. A. M. A., 1945, 128, 495.
3. Guttman, S. A., Potter, H. R., Hanger, F. M., Moore, D. B., Pierson, P. S., and Moore, D. H., Significance of cephalin-cholesterol flocculation test in malarial fever. J. Clin. Invest., 1945, 24, 296.
4. Read, H. S., Kaplan, L. I., Becker, F. T., and Boyd, M. F., An analysis of complications encountered during therapeutic malaria. Ann. Int. Med., 1946, 24, 444.
5. Mirsky, I. A., von Brecht, R., and Williams, L. D., Hepatic dysfunction in malaria. Science, 1944, 99, 20.
6. Kern, R. A., and Norris, R. F., Liver involvement in malaria. U. S. Nav. Med. Bull., 1944, 43, 847.
7. Lippincott, S. W., Ellerbrook, L. D., Hesselbrock, W. B., Gordon, H. H., Gottlieb, L., and Marble, H., Liver function tests in chronic relapsing *vivax* malaria. J. Clin. Invest., 1945, 24, 616.
8. Tumulty, P. A., Nichols, E., Singewald, M. L., and Lidz, T., An investigation of the effects of recurrent malaria; organic and psychological analysis of 50 soldiers. Medicine, 1946, 25, 17.
9. Neefe, J. R., Miller, T. B., and Chornock, F. W., Homologous serum jaundice. A review of the literature and report of a case. Am. J. M. Sc., 1944, 207, 626.
10. Neefe, J. R., Stokes, J. Jr., Reinhold, J. G., and Lukens, F. D. W., Hepatitis due to the injection of homologous blood products in human volunteers. J. Clin. Invest., 1944, 23, 836.
11. Neefe, J. R., Gellis, S. S., and Stokes, J., Jr., Homologous serum hepatitis and infectious (epidemic) hepatitis. Am. J. of Med., 1946, 1, 3.
12. Havens, W. P., Jr., Experiment in cross immunity between infectious hepatitis and homologous serum jaundice. Proc. Soc. Exp. Biol. & Med., 1945, 59, 148.
13. Solomon, M. H., King, A. J., Williams, D. I., and Nicol, C. S., Prevention of jaundice resulting from anti-syphilitic treatment. Lancet, 1944, 2, 7.
14. Sheehan, H. L., Epidemiology of infectious hepatitis. Lancet, 1944, 2, 8.
15. Stokes, J., Jr., and Neefe, J. R., The prevention and attenuation of infectious hepatitis by gamma globulin. J. A. M. A., 1945, 127, 144.
16. Havens, W. P., and Paul, J. R., Prevention of infectious hepatitis with gamma globulin. J. A. M. A., 1945, 129, 270.
17. Grossman, E. B., Stewart, S. G., and Stokes, J., Jr., Post-transfusion hepatitis in battle casualties and study of its prophylaxis by means of human immune serum globulin. J. A. M. A., 1945, 129, 991.

MECHANISM AND SIGNIFICANCE OF THE THYMOL TURBIDITY TEST FOR LIVER DISEASE

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An increase in the amounts of the globulin components of the serum has long been recognized in advanced states of liver disease (1, 2, 3), although the significance of the alteration has never been defined. It has been the basis of the Takata-Ara, Weltmann and the formol-gel reactions which have been used for many years in the diagnosis of liver disease. The nonspecific nature of these tests is now clear following the demonstration that positive reactions are found in any disease showing marked hyperglobulinemia (4, 5, 6). More recently, several new serum reactions, which appear to depend on small changes in the proteins of the serum, have been used for demonstrating liver disease. These include the cephalin flocculation, the colloidal gold and the thymol turbidity tests. The cephalin flocculation reaction has been studied in detail by Hanger and his coworkers (7, 8) and has been found to be a sensitive index of liver damage. The test may be positive in patients with liver disease who show normal serum protein values according to the usual methods of protein estimation and, conversely, the serum of patients with marked hyperglobulinemia may show negative cephalin flocculation reactions. The exact serum protein constituent that is altered in liver disease and is responsible for a positive cephalin flocculation test has not been clearly established; recent work by Hanger (8) appears to implicate albumin in addition to gamma globulin. The final solution to the problem is hampered by the complexity of the cephalin flocculation reaction, a fact which is also true of the colloidal gold reaction.

The technique of the thymol turbidity test is much simpler, however, and probably consists of a direct precipitation of a protein appearing in liver disease by the addition of a thymol solution. It would seem, therefore, that a study of the mechanism of this reaction and the protein component concerned would be more likely to yield clear-cut information regarding at least one of the proteins that appear in the blood stream during diseases of the liver. The present study was an attempt to elucidate the mechanism of the reaction and to define its significance in terms of clinical observations.

MATERIALS AND METHODS

The sera used in the study of the thymol turbidity reaction were selected from a group of 200 patients with infectious hepatitis and 65 patients with other liver disorders admitted to the Out Patient Department of the Hospital of the Rockefeller Institute.

Technique of performing the thymol turbidity test. The thymol reagent was prepared as described by Maclagan (9). Slight variations of this method produced unsatisfactory results. In alkaline solution thymol is somewhat unstable and turbidity of the reagent often occurs on standing. Exposure to air increases the turbidity of the solution and it was found important to keep the thymol reagent tightly stoppered. As the solution becomes increasingly cloudy, its activity decreases and it is important that only clear or very slightly turbid solutions be used. If properly prepared, the thymol reagent is satisfactory for at least one month. Although the pH of the thymol reagent was slightly lower than that originally stated by Maclagan, it proved to be satisfactory.

Three ml. of the thymol reagent were added to 0.05 ml. of serum and the degree of turbidity measured in the Coleman Jr. spectrophotometer at 650 mμ. This represented a 1/60 dilution and corresponded to that originally described by Maclagan. Figure 1 shows the turbidity of sera from cases of liver disease at various dilutions of reagent using saline dilutions as controls. It can be seen that the maximum turbidity was usually obtained at a 1/12 dilution and that differences in certain sera at the lower dilution might not be so marked at the 1/60 dilution. Although the use of lower dilutions has certain apparent advantages, all determinations discussed in this paper were performed at Maclagan's standard 1/60 dilution.

The degree of turbidity was compared with a $BaSO_4$ standard as described in a previous publication (10). This proved to be a satisfactory standard regardless of tube size or type of instrument used. The units of turbidity corresponded to those originally described by Maclagan using visual comparators.

[1] Deceased, August 2, 1946.

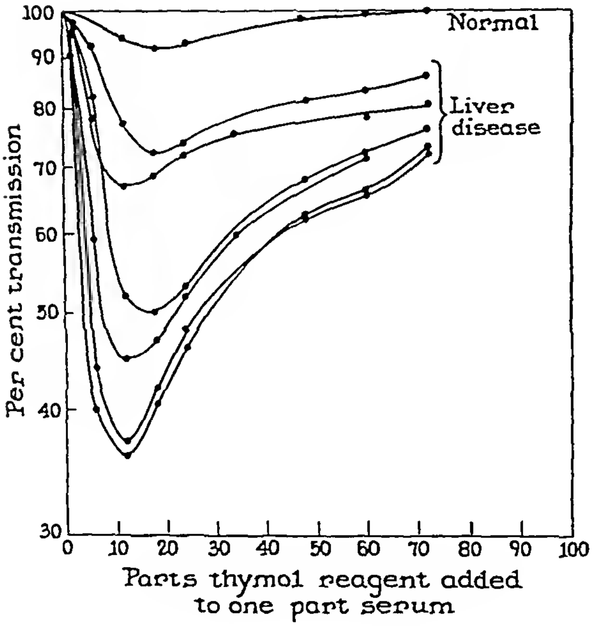

FIG. 1. TURBIDITY READINGS AS MEASURED IN THE SPECTROPHOTOMETER AT VARIOUS DILUTIONS OF SERUM WITH THYMOL REAGENT

Estimation of the degree of flocculation of the thymol serum mixture was also carried out in a number of patients. The finding of Neefe (11) that in certain patients, following an attack of infectious hepatitis, the 24-hour flocculation with thymol reagent remained positive for a slightly longer period than the usual thymol turbidity reaction, was confirmed. In general, however, the estimation of flocculation proved to be a less sensitive index than turbidity determinations, and did not furnish quantitative results.

Electrophoretic analyses were made in diethylbarbituric acid buffer ($\mu = 0.1$, pH $= 8.6$) by the method of Longsworth (12). Determinations of lipids were carried out by the gasometric lipid carbon method of Van Slyke and Folch (13). Total and free cholesterol of the plasma was determined by the method of Schoenheimer and Sperry (14). Extraction of lipids from serum was done by freezing in the presence of ether as described by McFarlane (15). A modification of Hanger's method (7) was used for the determination of the cephalin flocculation reaction. Bromsulfalein retention was estimated by the method of Rosenthal and White (16) modified for the use of the Coleman Jr. spectrophotometer. Globulin was determined electrophoretically and by fractionation with Na_2SO_4 (17). In addition, quantitative measurements of globulin were obtained by a turbidometric technique (18).

Immunological experiments were carried out by injecting rabbits with 5 to 8 mgm. of thymol protein every 2 days for 8 injections. The antiserum was absorbed with normal human serum. Precipitin tests were carried out by the technique of Swift, Wilson, and Lancefield (19).

EXPERIMENTAL

I. *The relation of the thymol turbidity reaction to the lipids in the serum*

It was demonstrated by Maclagan (9) that the precipitate resulting from the addition of thymol reagent to serum is high in cholesterol and phospholipids. Recant, Chargaff and Hanger (20) found that sera from cases of liver disease from which lipids had been extracted with ether no longer showed turbidity following the addition of thymol reagent. These observations indicated that lipids are an important factor in the thymol turbidity reaction. This work was confirmed and extended. Thymol was found to have a special effect on lipids in general. Any lipid emulsion tended to be broken up by the addition of a thymol solution. Figure 2 shows the effect of thymol in increasing the particle size of a lipid emulsion as viewed under the microscope. When such emulsions were visualized with the naked eye, an increase in turbidity accompanied the change in particle size. This turbidity was purely the result of the physical alteration in the lipid emulsion resulting from the addition of thymol. Lipemic sera from patients with liver disease, nephrosis, diabetes, and thyroid disease all showed an increase in turbidity upon the addition of the thymol reagent. This, however, was purely a physical change in the lipid emulsion, since no protein was precipitated as in the usual thymol turbidity reaction accompanying liver disease. The following example serves to illustrate this point.

Three-tenths ml. of lipemic serum from a patient with nephrosis was diluted 60 times with the thymol barbital buffer reagent. This gave an increased turbidity over

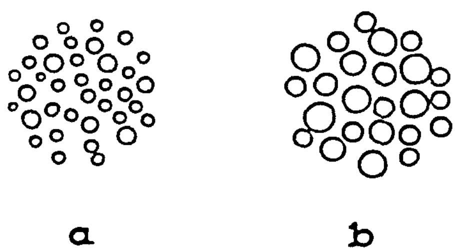

FIG. 2. COMPARISON OF THE PARTICLE SIZE OF EQUAL QUANTITIES OF A LIPID SUSPENSION IN (a) BARBITAL BUFFER, (b) BARBITAL BUFFER PLUS THYMOL, AS VIEWED UNDER THE MICROSCOPE

the serum with barbital buffer alone equivalent to 60 units. The turbid mixture was then spun for 3 hours in the centrifuge at 15,000 r.p.m. No sediment settled at the bottom of the tube, but a white layer formed at the surface leaving a clear solution underneath. Analysis of the surface layer showed that it contained 6 mgm. total lipid and 0.001 mgm. nitrogen. The material responsible for the turbidity had been brought to the surface of the solution by high speed centrifugation and was found to contain a negligible amount of protein. A similar experiment carried out on the turbid material resulting from the addition of thymol reagent to clear serum from a case of infectious hepatitis demonstrated that the cloudy material all settled to the bottom of the tube. Nitrogen analysis of the precipitate showed that it contained 0.44 mgm. of N. Whereas the turbidity of this serum had been found to be equivalent to only 30 units, the material responsible for the turbidity consisted of a large amount of protein.

It appears, therefore, that the turbidity produced by the action of the thymol reagent on lipemic sera from subjects without liver disease is due to an increase in the particle size of the protein-free lipid suspension, while the turbidity produced by the reagent in clear hepatitis serum is due to the formation of a protein-lipid-thymol complex.

Since certain sera from cases of liver disease are lipemic, it was of some importance to find a simple method of determining how much of the turbidity produced by the thymol reagent indicated a true reaction with precipitation of protein. When a thymol solution having the same pH as the usual reagent, but with a high ionic strength, was added to clear serum from a patient with liver disease giving a positive reaction, no turbidity appeared. However, when this solution was added to an artificial lipid emulsion or lipemic sera from patients with nephrosis, the turbidity produced was the same as that caused by the low ionic strength reagent. The thymol altered the state of the lipids regardless of the ionic strength of the solution. The usual protein precipitation reaction occurred only with the low ionic strength thymol reagent. In evaluating the turbidity produced in lipemic serum by Maclagan's thymol reagent, the amount of turbidity due to protein precipitation alone was obtained by using as blank in the photometer serum with the high ionic strength thymol buffer (Table I).

The presence of lipids in the serum is also an essential factor in the usual thymol turbidity reaction, as indicated by the fact that positively reacting sera after extraction with ether no longer give the reaction. The essential role of the lipids was further borne out by the finding that surface-acting agents, such as various tweens, completely inhibited the formation of any precipitate in hepatitis serum to which thymol had been added. Once formed, the thymol precipitate also dissolved readily on the addition of small amounts of tween 80.[2] In other protein precipitation reactions which depended purely on the natural solubility of the proteins involved, the presence of tween actually enhanced the precipitation. The action of the tween was undoubtedly related to its effect on the state of the lipids involved in the reaction.

Since the soluble lipids play an essential part in the thymol turbidity reaction, it seemed important to test the effect of the addition of various concentrations of lipid on the reaction. Because of the specific effect of thymol on lipid suspensions it was important to keep the lipids in their most soluble state. As a result, lipid was added in the form of clear serum giving a negative thymol turbidity reaction. Four such sera were chosen containing varying amounts of lipid. When each of these sera was added to normal serum, the thymol turbidity reaction of the combination remained negative. However, when added to serum with a high gamma globulin level giving a positive thymol reaction, the turbidity of the combination was proportional to the amount of lipid in the added serum. This effect was more strikingly brought out by first extracting the

TABLE I

Comparison of the turbidity in units obtained in the spectrophotometer for various sera upon the addition of the thymol reagent, using as the zero control the same sera with (a) buffer alone, (b) high ionic strength buffer with thymol

Type of serum	Buffer at pH 7.6 and $\mu = .01$	Buffer + thymol at pH 7.6 and $\mu = 1.0$
Clear serum from patients with infectious hepatitis	26	26
Lipemic serum from patient with nephrosis	52	0
Lipemic serum from patient with infectious hepatitis	28	16

[2] Polyoxyethylene sorbitan monooleate.

lipids from the serum of another patient with a high gamma globulin level in the serum. The thymol turbidity reaction was reduced from 22 to 9 units by this procedure. Table II shows the relative effectiveness of various sera in restoring a positive thymol turbidity reaction to this extracted hepatitis serum. It can be seen that the high lipid sera had a much greater effect than normal serum. The major portion of the increased turbidity was due to increased precipitation of lipid as shown by the protein and lipid analyses of the precipitates. Figure 3 demonstrates more clearly the comparative effect of various lipid concentrations on the turbidity, protein content, and lipid content of the precipitate formed with extracted hepatitis serum. The results were obtained in the same experiment illustrated in Table II. It is evident that, although there is increased precipitation of protein in the presence of higher lipid concentrations, the major portion of the increased turbidity is due to increased precipitation of lipid. In other words, the resulting turbidity reflected primarily the concentration of lipid in the added serum. The relationship was so close that it was possible to use this system as a rapid method of estimating the concentration of lipid in an unknown serum.

TABLE II

Relative effect of the addition of 0.2 ml. of sera, differing only in their lipid content, in restoring a positive thymol turbidity reaction to 0.2 ml. ether-extracted hepatitis serum

Lipid content of serum added	Thymol turbidity of serum added	Thymol turbidity of combination of extracted hepatitis serum and added serum	Protein in precipitate	Lipid in precipitate
mgm. per cent	*units*	*units*	*mgm.*	*mgm.*
500	3	14	1.84	1.11
900	4	27	2.58	2.46
2000	4	50	3.41	5.22

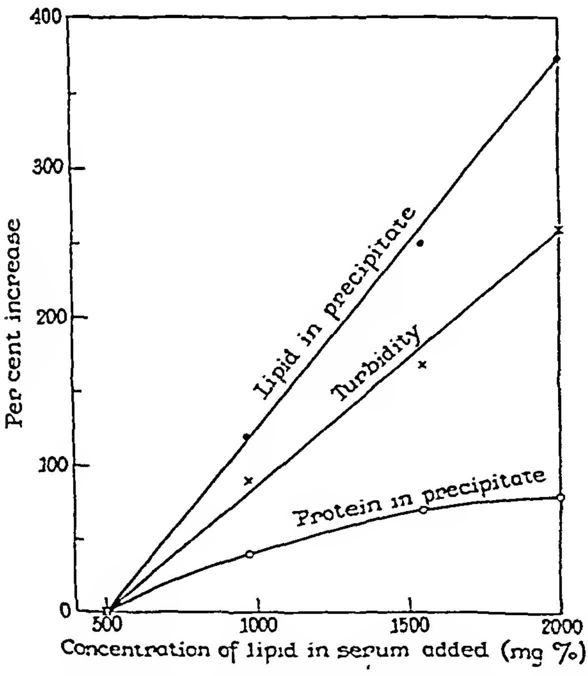

FIG. 3. EFFECT OF THE ADDITION OF SERA CONTAINING VARIABLE AMOUNTS OF LIPID ON THE TURBIDITY, LIPID CONTENT, AND PROTEIN CONTENT OF THE PRECIPITATE FORMED IN EXTRACTED HEPATITIS SERUM WITH THYMOL REAGENT

The above data demonstrated the marked influence of lipid concentration on the thymol turbidity reaction in a somewhat artificial system involving the addition of sera with variable lipid concentrations. In order to evaluate more clearly the effect of the concentration of lipids in sera on the reaction as it is usually applied, lipid analyses were carried out simultaneously on the serum of patients with liver disease and the specific precipitate resulting from the addition of thymol reagent. The amount of lipid in the precipitate varied from 20 to 50 per cent and was directly proportional to the concentration of lipid in the original serum (Figure 4). The turbidity that is usually measured in the thymol turbidity reaction depends on both the protein and lipid that are precipitated. Since the concentration of lipid in the precipitate is proportional to the concentration in the serum, it is clear that the thymol turbidity reaction actually determines in part the concentration of lipid in the serum of patients with liver disease.

Despite the fact that the level of the lipids in the serum is one of the variables that is measured in the thymol turbidity reaction, a number of patients with liver disease other than infectious hepatitis have been observed with high lipid levels in the serum but with low or negative thymol turbidity test values. Fractionation of the total lipid in these cases into the cholesterol and phospholipid partitions did not reveal any specific effect. The addition of reactive protein, or serum contain-

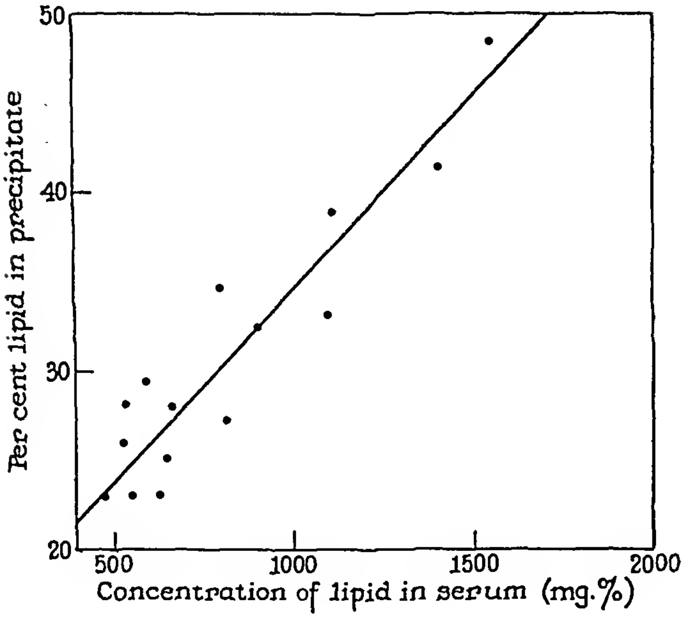

FIG. 4. THE RELATION OF THE LIPID CONTENT OF SERUM FROM PATIENTS WITH LIVER DISEASE TO THE LIPID CONTENT OF THE SPECIFIC PRECIPITATE FORMED IN THE THYMOL TURBIDITY REACTION

ing reactive protein, always produced a positive reaction in proportion to the total lipid concentration. The presence of the thymol-reacting protein was the essential factor while the level of the lipids affected only the intensity of the reaction.

II. *Identification of the protein involved in the thymol turbidity reaction*

Maclagan (9) analyzed the precipitate obtained in a positive thymol turbidity reaction and found that it consisted of approximately 40 per cent protein. He suggested that this was probably a gamma globulin because of its low solubility. Hanger and associates (20) were unable to obtain positive thymol turbidity reactions by adding gamma globulin electrophoretically separated from hepatitis sera to normal sera and various lipid fractions. They concluded that the protein involved in the reaction was probably an alpha or beta globulin and not in the gamma globulin fraction.

(*a*) *Electrophoretic analysis of the thymol precipitate.* Studies of the precipitate obtained with thymol reagent were hampered by the large amount of lipid that was present. Turbid solutions were always obtained when attempts were made to dissolve the precipitate and it was impossible to obtain an electrophoretic pattern. Extraction of the lipids from the precipitate in the cold with ether alone, ether and alcohol, or acetone alone, always resulted in denaturation of the proteins in the precipitate. When removed from serum the proteins were apparently less resistant to the action of organic solvents.

Attempts were also made to dissolve the precipitate resulting from the thymol reaction in normal serum and observe the change in the electrophoretic pattern. The high lipid concentration, however, still interfered and no conclusive results could be obtained. The difficulty was finally overcome by the use of tween 80, a strong emulsifying agent. A 1 per cent concentration of this material did not affect the electrophoretic pattern of normal serum. A clear solution suitable for electrophoretic determinations was obtained in the following manner.

Two hundred ten ml. of thymol reagent were added to 15 ml. of very reactive hepatitis serum (38 units) at 0° C. The precipitate was collected by centrifugation in the cold and suspended in 10 ml. barbital buffer containing 1 per cent tween at pH 8.5 and $\mu = 0.1$. This was then dialyzed in the cold against barbital buffer at the same pH and ionic strength. A small amount of sediment that still remained was thrown down by centrifugation and the resulting supernate was quite clear.

Figure 5 shows the electrophoretic pattern of the protein solution obtained in the above manner. The sharp peak (*b*), representing almost the entire amount of protein present, had a mobility of 1.6×10^{-5} which places it in the gamma globulin fraction but with an unusually rapid mobility. A

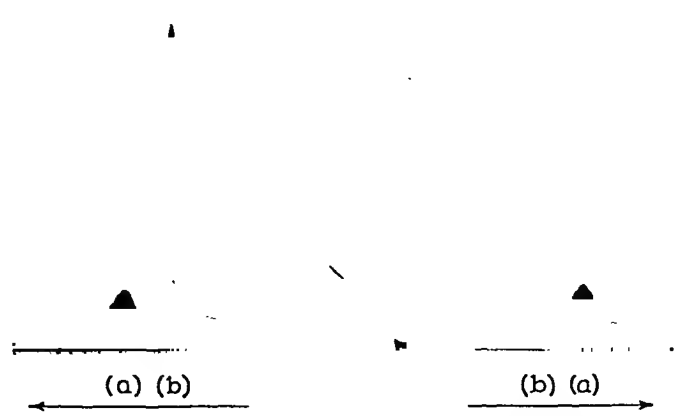

FIG. 5. ASCENDING AND DESCENDING ELECTROPHORETIC PATTERNS OF THE PROTEIN COMPONENT OF THE PRECIPITATE FORMED IN THE THYMOL TURBIDITY REACTION

Peak (*a*) $= \beta$ globulin ($\mu = 2.83 \times 10^{-5}$); peak (*b*) $= \gamma$ globulin ($\mu = 1.60 \times 10^{-5}$).

small definite beta globulin peak (*a*) is also visible. In a second experiment with material from another patient, lipid and tween were removed by subjecting the protein to repeated precipitations from large volumes of solution by lowering the salt concentration. The final preparation contained no lipid and the electrophoretic pattern showed a single peak with the mobility of a gamma globulin. Further experiments with the thymol-precipitated protein in the absence of tween indicated that when all of the lipid was removed by centrifugation at 12,000 r.p.m. at high salt concentrations, the remaining protein migrated extremely slowly. In 1 experiment the mobility was 0.5×10^{-5}. It appeared as if the more lipid that was removed, the more slowly the remaining protein migrated.

(*b*) *Effect of the addition of gamma globulin to normal serum.* The importance of the gamma globulin fraction was borne out in a series of experiments with gamma globulin separated electrophoretically from various types of sera. Considerable difficulty was encountered in adjusting the protein concentration, the ionic strength, and the pH of each fraction to exactly the same level because of the small and variable amount of gamma globulin that was obtained by electrophoretic separation. Slight variation in these fractions caused marked differences in their effect on the thymol turbidity reaction of serum and, as a result, this method of studying the protein component responsible for the reaction was not completely satisfactory. However, it was possible to demonstrate that gamma globulin fractions from certain sera demonstrating a very high thymol turbidity reaction, although negative alone, produced positive reactions with thymol reagent when added to normal serum. The gamma globulin precipitated with thymol reagent only in the presence of lipid. The amount of turbidity produced was proportional to the concentration of lipid in the serum to which the gamma globulin was added. No turbidity was produced with serum in the presence of a small amount of tween.

That the gamma globulin fraction is important in the thymol reaction as it is usually carried out was also indicated by experiments where this fraction was removed from 2 positive sera electrophoretically. The sera, after being brought to their previous state in all respects, except that the gamma globulin was absent, now gave a negative reaction.

Comparison of gamma globulin preparations from various sera in respect to their ability to induce a positive reaction in the presence of lipid was attempted (Table III). Four mgm. of gamma globulin were added to each 0.1 ml. of serum used. The gamma globulin obtained from the serum showing the pattern illustrated in Figure 6*a* was slightly more active in inducing a positive thymol reaction than was that obtained from normal serum. It also appeared to be slightly more active than was gamma globulin obtained from the serum of a patient with cirrhosis of the liver which showed an increase in the gamma globulin fraction but a negative thymol turbidity reaction. A definite and clear-cut difference in activity was obtained when the hepatitis gamma globulin was compared with gamma globulin from the serum of a patient with multiple myeloma. The latter serum had given a negative thymol turbidity reaction. Since all the gamma globulin preparations had some activity in the presence of lipid, it was impossible to draw very definite conclusions. Certainly, the activity of the gamma globulin preparations was not proportional to the activity of the sera from which they were obtained.

TABLE III

Comparative effect of equal concentrations of gamma globulin, obtained from various sources, on the thymol turbidity reaction of a high lipid serum

Source of gamma globulin	Thymol turbidity of serum	Thymol turbidity of gamma globulin on addition of serum containing 950 mgm. per cent lipid
	units	*units*
1. Hepatitis serum	40	26
2. Hepatitis serum	31	28
3. Normal serum	3	16
4. Normal serum	2	20
5. Cirrhosis serum	4	21
6. Multiple myeloma serum	3	8

(*c*) *The change in the electrophoretic pattern of hepatitis serum following removal of the thymol protein.* In order to obtain further information about the protein or protein complex that is precipitated with thymol reagent, electrophoretic analyses were carried out on 3 highly active hepatitis sera before and after removal of the thymol

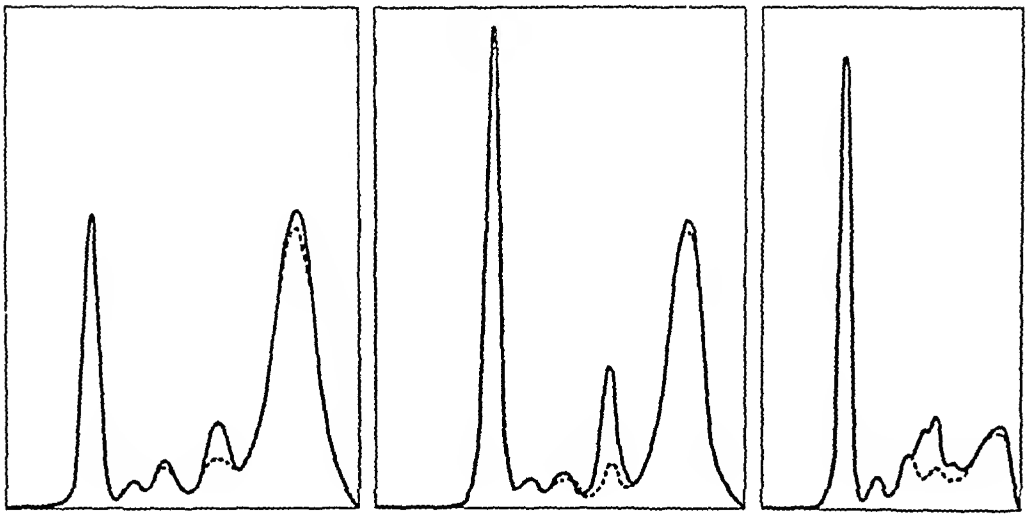

FIG. 6. ASCENDING ELECTROPHORETIC PATTERNS BEFORE AND AFTER REMOVAL OF THE THYMOL PRECIPITATE IN 3 PATIENTS DEMONSTRATING VERY HIGH VALUES FOR THE THYMOL TURBIDITY TEST

The dotted lines indicate the areas which were altered by the precipitation.

precipitate. The thymol precipitate-free serum was obtained in the following manner.

To 7 ml. of hepatitis serum, 98 ml. of thymol reagent were added. The precipitate was permitted to settle for 30 minutes and then removed by centrifugation. The clear supernatant solution was dialyzed against saline until all traces of thymol were removed. The volume of solution was brought down by evaporation in the dialysis bag placed in front of an electric fan. This procedure was continued until the protein content of the solution was equal to that in the original serum minus the quantity in the thymol precipitate. Electrophoretic analyses were then carried out after the usual adjustment to a pH of 8.6 and $\mu = 0.1$ by dialysis against barbital buffer.

By superimposing the tracings obtained before and after removal of the thymol precipitate (Figure 6), the differences in the electrophoretic patterns of the 3 sera are clearly visible. The predominant alteration was in the beta globulin fraction. The question arises as to whether the change in this fraction is due solely to the removal of lipid from the serum as a result of the thymol precipitation. A decrease in the beta globulin is known to occur with extraction of lipids from the serum by means of organic solvents. Calculation by planimetry of the protein lost from the gamma globulin peak showed some variation in the ascending and descending patterns of the same serum. In addition, the amount of protein lost by the precipitation was small in comparison with the total protein, less than 8 per cent in all 3 cases. It was, therefore, difficult to obtain exact evidence as to how much of the protein precipitated came from the gamma globulin fraction. The figures obtained ranged between 25 and 45 per cent with an average of 37 per cent for the ascending and descending patterns of the 3 sera. These results indicated a portion of the precipitated protein came from the gamma globulin fraction but that the largest portion came from the beta fraction.

(*d*) *Immunological results.* In order to find out more definitely whether the globulin precipitated in the thymol turbidity reaction is an abnormal protein or just an increased amount of normal protein in the serum, antibodies to the protein illustrated in Figure 5 were obtained in rabbits. The antiserum reacted strongly with normal serum, and no definite difference could be obtained with sera containing large amounts of gamma globulin or sera giving a very positive thymol turbidity reaction. Absorption of the antiserum with normal serum did not aid in demonstrating a difference between normal and hepatitis serum.

(*e*) *Effect of albumin on the thymol turbidity reaction.* In view of the known effect of albumin in increasing the solubility of proteins in general and the specific effect of albumin on the cephalin flocculation reaction (8), studies were carried out to determine its effect on the thymol turbidity reaction. Figure 7 demonstrates the fall in the values

for the thymol turbidity test in the serum of a patient with hepatitis upon the addition of increasing amounts of concentrated human albumin. The albumin concentration of the serum had to be almost doubled before much change occurred.

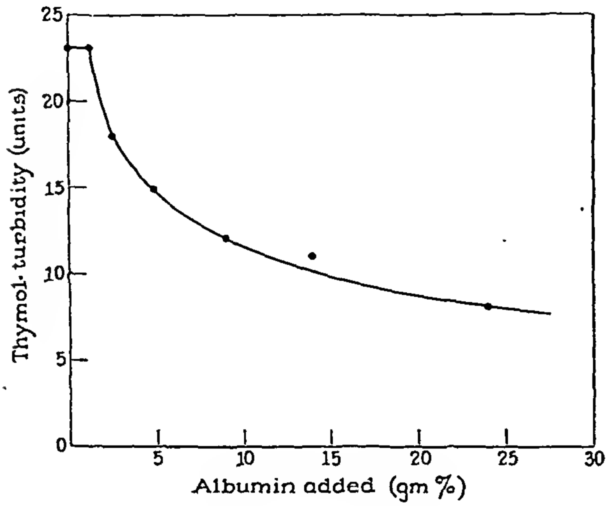

FIG. 7. THE EFFECT OF THE ADDITION OF VARIOUS AMOUNTS OF HUMAN ALBUMIN ON THE THYMOL TURBIDITY REACTION IN THE SERUM OF A PATIENT WITH INFECTIOUS HEPATITIS

In vivo studies with concentrated solutions of human serum albumin in patients with diseases of the liver have shown a definite decrease in the intensity of the thymol turbidity reaction of the serum following intensive therapy with this material. The decrease in the thymol turbidity reaction could be accounted for partly on the basis of an increase in plasma volume and on the *in vitro* effect of albumin described in the previous paragraph. However, in certain patients the fall was too great to be explained in such a manner. An example of such an effect was seen in the case of a 12-year-old boy with very severe acute infectious hepatitis accompanied by edema and ascites. The administration of three 25-gram units of albumin produced a fall in the thymol turbidity reaction from 27 to 7 units with an increase of only 1 gram per cent in the serum albumin level. The change in plasma volume in this patient was approximately 10 per cent. The cephalin flocculation reaction showed no change during this period. The thymol turbidity reaction returned to its original level within 6 days following cessation of therapy. Apparently, in the body, albumin may have a specific effect on the reaction not encountered in the *in vitro* experiment. The level of albumin in the body, therefore, is another factor influencing the intensity of the thymol turbidity reaction.

III. *Clinical observations on the effect of the protein and lipid components of the serum on the thymol turbidity reaction during the course of acute infectious hepatitis.*

In order to demonstrate further the influence of the lipid level of the serum on the reaction under study, lipid determinations were carried out on 2 groups of patients during the early icteric phase of acute infectious hepatitis (Table IV). The

TABLE IV

Average lipid concentration in the serum of patients with acute infectious hepatitis showing high thymol turbidity test values compared with those showing low values

Group	No. of patients	Thymol turbidity test values	Average total lipid concentration
A	6	above 25	1275
B	6	below 15	610

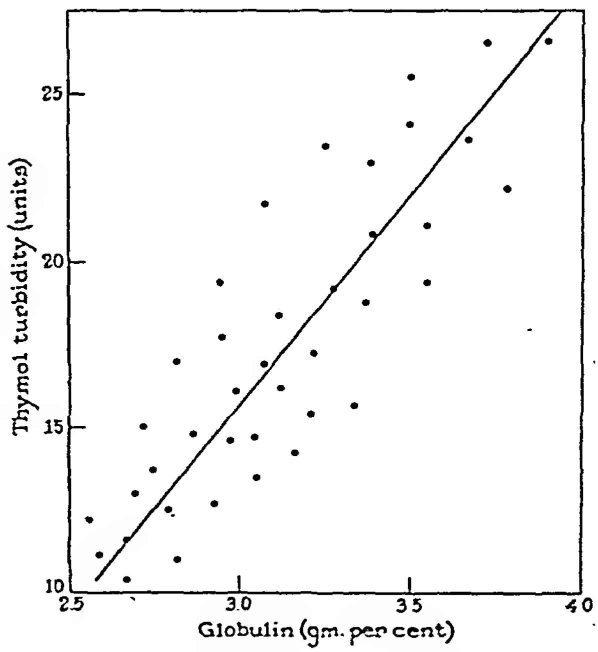

FIG. 8. RELATION BETWEEN THE ELEVATION OF THE THYMOL TURBIDITY TEST AND THE GLOBULIN LEVEL OF THE SERUM DURING THE CONVALESCENT PERIOD OF ACUTE INFECTIOUS HEPATITIS

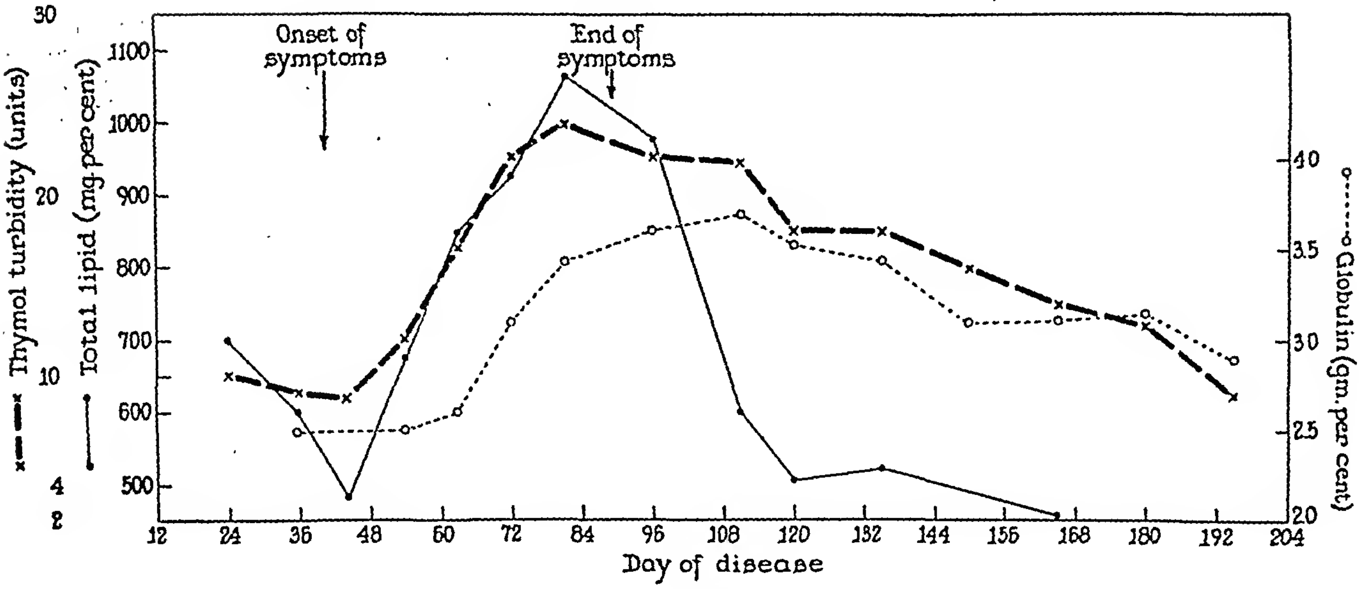

FIG. 9. ILLUSTRATION OF THE CLOSE PARALLELISM BETWEEN THYMOL TURBIDITY AND LIPID DETERMINATIONS INITIALLY, AND BETWEEN THYMOL TURBIDITY AND TOTAL GLOBULIN DETERMINATIONS DURING THE LATER PERIOD IN A RELAPSE OF INFECTIOUS HEPATITIS

severity of the illness in the 2 groups was approximately equal and they differed only in the intensity of the thymol turbidity reaction. None of the patients showed more than slight elevation of the globulin level. It can be seen that the average lipid concentration in the sera of patients with high values for the thymol turbidity test was much greater than in the group showing low values.

During the convalescent period of infectious hepatitis the influence of the lipid level became less pronounced and values for the thymol turbidity test paralleled the globulin level of the serum to a close degree. Figure 8 demonstrates this relation in a group of patients during convalescence at a time when the lipid changes were minimal.

The comparative influence of the protein and lipid components on the thymol turbidity reaction during the course of infectious hepatitis may also be seen from Figure 9. This chart illustrates the close parallelism of values obtained by simultaneous determinations of the thymol turbidity reaction and plasma lipids in demonstrating a delayed rise during the course of a recurrence of acute infectious hepatitis. The total globulin level showed an even more delayed elevation and during the late convalescent period the thymol turbidity reaction followed the pattern of the globulins very closely. Electrophoretic determinations demonstrated that the rise in total globulin was almost entirely due to an increase in the gamma globulin fraction.

DISCUSSION

The experiments that have been described make it clear that there are 2 main components in serum that are important in the thymol turbidity reaction: (*a*) the protein component, (*b*) the lipid component. Electrophoretic analyses have indicated that these 2 components are not separate but are intimately related. The entire question revolves about the difficult problem of loose protein-lipid combinations that migrate in the beta globulin fraction of serum. The ease with which these combinations are broken makes it difficult to study the lipid-protein complexes individually. The observations reported have shown a slower electrophoretic migration of the protein as the lipid is removed and suggest the possibility that some beta globulins may represent light combinations of lipid and gamma globulin.

In view of the specific effect of thymol on lipids in general, it seems logical to suppose that dilution of serum with a thymol solution would tend to throw lipid-protein complexes out of solution. The protein is carried along by the effect of thymol in decreasing the solubility of the lipids. In addition, the low ionic strength buffer used in the reagent will sometimes precipitate gamma globulin

from hepatitis serum (18) in the absence of thymol. Such a buffer solution has been used by Wolff for detecting the globulin elevation in malaria serum (21). The effect of the thymol in increasing the precipitate appears to be attributable to the action of thymol in decreasing the emulsification of lipids. The inhibitory effect of emulsifying agents on the thymol turbidity reaction also indicated that the protein precipitation was enhanced by the effect of thymol on the lipids in the serum. The reaction, therefore, probably results from a combination of the effect of thymol in decreasing the dispersion of the lipid component, and the effect of dilution with low ionic-strength buffer in decreasing the solubility of the globulin component, in such a way as to precipitate the protein-lipid combination.

The fact that the lipids of the serum play a greater role in the thymol turbidity reaction than just forming a part of the protein-lipid-beta globulin complex essential to the reaction, is indicated by the observations which demonstrated that the per cent lipid in the thymol precipitate is proportional to the total lipid in the serum of patients with infectious hepatitis. In addition, the experiments with mixtures of 2 different sera showed that a high lipid serum, although negative by itself, markedly affected the intensity of the reaction of the combination.

The effect of gamma globulin in acting with normal serum to give a positive thymol turbidity reaction suggests a similarity to the cephalin flocculation reaction where such an effect is also seen (8). One of the main points of dissimilarity between the 2 reactions that has been cited (20) is that extraction of the lipids from hepatitis serum does not affect the cephalin flocculation reaction as it does the thymol reaction. This, however, cannot be considered a fundamental difference because, in the cephalin flocculation reaction, the cephalin-cholesterol reagent used supplies ample lipid to an extracted serum. The thymol alters the state of the lipids of the serum so that a lipid globulin complex precipitates from the serum of certain patients with liver involvement. In the cephalin flocculation reaction, a lipid suspension is added to the serum so that a lipid globulin complex precipitates. The difference in the 2 tests is mainly in the manner in which the lipid globulin precipitation is brought about. Clinically, the 2 tests are related, although occasional marked differences do occur (11, 22 to 24). One of the main discrepancies is seen in the earlier development of a positive cephalin flocculation reaction during the pre-icteric stage of acute infectious hepatitis. A possible explanation may lie in the fact that the lipids show a delayed rise in this disease, and, therefore, the effect of increased lipids on the thymol reaction would not be present.

An increased concentration of gamma globulin was found electrophoretically in all sera showing a positive thymol turbidity reaction. Most of the positive sera but not all also showed increased amounts of beta globulin. There is difficulty in explaining negative reactions in certain patients with cirrhosis of the liver who have markedly increased gamma globulin levels, high beta globulin concentrations, high total lipid values and, in addition, low plasma albumin. This should be the ideal situation for a very positive thymol turbidity reaction. The evidence for a qualitative difference in the increased globulin is not sufficiently conclusive to explain such a case entirely, especially in view of the negative results obtained in immunization experiments. Similar discrepancies have been noted in the cephalin flocculation reaction and Hanger and his associates (8) have obtained evidence that albumin plays an important role. This problem requires further study.

In the course of studies on patients with various types of liver disease associated with marked hypergammaglobulinemia, it was noted that most of them had equally high thymol turbidity values: approximately 35 units. This was also true of sera from patients with kala azar and schistosomiasis with marked hypergammaglobulinemia. This represented a protein precipitation of approximately 0.8 gram, a very small portion of the gamma globulin of these sera. This was not true of patients with multiple myeloma. The gamma globulin elevation in this disease appears to be very different. A negative thymol turbidity reaction was always obtained. It appeared as if there was a maximal amount of protein that could be precipitated. A possible explanation for this phenomenon may reside in the fact that all these sera showed approximately the same amount of lipid and, therefore, only a limited amount of lipid was

available to precipitate with globulin in the presence of thymol reagent. More protein readily precipitated if additional lipid was added.

The close relation between the level of the lipids in serum and the intensity of the thymol turbidity reaction must be kept in mind in evaluating observations by this test because of the frequent occurrence of elevation of the plasma lipids in liver disease. The dependence of the test on 2 variable factors, the protein and lipid components, is somewhat of a disadvantage to a clear analysis of the significance of the reaction. This may be the reason for the lack of correlation between the intensity of the reaction and the severity of illness observed in a clinical study of infectious hepatitis (22).

Serial determinations of the thymol turbidity test during the course of infectious hepatitis have revealed that values for the test show a delayed rise following the onset of the disease and a prolonged elevation during convalescence (25). The relative effect of the lipid and globulin components of the reaction upon this pattern was demonstrated. The values for the thymol turbidity test closely paralleled the plasma lipids during the acute phase of the disease and the total globulin level during convalescence. The prolonged high values for the thymol turbidity test following infectious hepatitis were due to persistent elevation of the serum globulins. These observations were substantiated by electrophoretic patterns taken during the course of this disease. During the early period of elevation of values for the thymol turbidity test the dominant abnormality was an increase in the electrophoretic beta globulin. This was a reflection of the elevated plasma lipids. Later the predominant aberration was an increase in the gamma globulin, which remained elevated as long as the thymol turbidity reaction remained positive. Thus, it is apparent that the thymol turbidity test has a different significance during different stages of a single disease such as acute infectious hepatitis.

Determination of the lipid changes alone is not a sensitive index of acute liver damage, and there is no simple specific method of determining the small increases in globulin following such damage. The thymol turbidity test in reflecting the combination of these aberrations is a more sensitive index of liver injury than either one alone. Since the essential factor in the mechanism of the reaction is the increase in the globulin component following acute liver injury, investigations of the significance of the reaction really call for an understanding of the corresponding hyperglobulinemia.

The serum globulins are known to show a delayed rise in several diseases; this has been considered to be a reflection of the development of antibodies. Recent studies on typhus (26), however, have demonstrated that complement fixation tests and the Weil-Felix reaction did not parallel the alteration in globulin. The explanation for the change in liver disease is also very obscure. Liver biopsies obtained from the patients with marked hyperglobulinemia have revealed marked evidence of regeneration of liver tissue, suggesting that globulin elevation may be correlated with the healing process.

SUMMARY

1. The turbidity produced by the thymol reagent of Maclagan in the serum of patients with infectious hepatitis is shown to depend on the presence both of lipids and of abnormal lipid protein complexes migrating in the beta globulin fraction of the serum. The gamma globulin fraction of serum also plays an important role in the reaction. The relative importance of the different components in the reaction varies with different sera. Development of the turbidity is prevented if the lipids are kept in solution by the addition of a tween or are extracted with ether, or if the gamma globulin is removed.

2. In lipemic sera the thymol reagent causes a nonspecific increase in the turbidity due to increase in particle size of the lipid globules. Correction for this effect can be made by using a photometric blank prepared from serum and a thymol reagent containing such a high buffer concentration that precipitation of the globulin component is prevented.

3. Immunological studies did not reveal evidence that the protein concerned in the reaction was abnormal.

4. A possible similarity between the mechanism of the thymol turbidity and cephalin flocculation reactions was discussed.

5. In patients with acute infectious hepatitis values for the thymol turbidity test were found to parallel alterations in serum lipids initially and alterations in gamma globulin during late convalescence.

BIBLIOGRAPHY

1. Filinski, W., L'Augmentation de taux de la globuline dans le sérum du sang comme résultat de l'insuffisance hépatique. Presse Méd., 1922, 30, 236.
2. Wiener, H. J., and Wiener, R. E., Plasma proteins. Arch. Int. Med., 1930, 46, 236.
3. Foley, E. F., Keeton, R. W., Kendrick, A. B., and Darling, D., Alterations of serum protein as an index of hepatic failure. Arch. Int. Med., 1937, 60, 64.
4. Cassiano P., Applicazioni della reazione di Takata in psichiatria. Schizofrenie, 1935, 4, 151.
5. Magath, J. B., The Takata-Ara test of liver function. Proc. Staff Meet., Mayo Clin., 1935, 10, 493.
6. Levinson, S. A., Klein, R. I., and Rosenblum, P., The Weltmann serum coagulation reaction; Preliminary report. J. Lab. & Clin. Med., 1937, 23, 53.
7. Kabat, E. A., Hanger, F. M., Moore, D. H., and Landow, H., The relation of cephalin flocculation and colloidal gold reactions to the serum proteins. J. Clin. Invest., 1943, 22, 563.
8. Moore, D. B., Pierson, P. S., Hanger, F. M., and Moore, D. H., Mechanism of the positive cephalin-cholesterol flocculation reaction in hepatitis. J. Clin. Invest., 1945, 24, 292.
9. Maclagan, N. F., The thymol turbidity test as an indicator of liver dysfunction. Brit. J. Exper. Path., 1944, 25, 234.
10. Shank, R. E., and Hoagland, C. L., A modified method for the quantitative determination of thymol turbidity reaction of serum. J. Biol. Chem., 1946, 162, 133.
11. Neefe, J. R., Results of hepatic tests in chronic hepatitis without jaundice; correlation with the clinical course and liver biopsy findings. Gastroenterology, 1946, 7, 1.
12. Longsworth, L. G., Recent advances in the study of proteins by electrophoresis. Chem. Rev., 1942, 30, 323.
13. Van Slyke, D. D., and Folch, J., Manometric carbon determination. J. Biol. Chem., 1940, 136, 509.
14. Schoenheimer, R., and Sperry, W .M., A micromethod for the determination of free and combined cholesterol. J. Biol. Chem., 1934, 106, 745.
15. McFarlane, A. S., Behavior of the lipoids in human serum. Nature, 1942, 149, 439.
16. Rosenthal, S. M., and White, E. C., Clinical application of the bromsulphalein test for hepatic function. J. A. M. A., 1925, 84, 1112.
17. Howe, P. E., The use of sodium sulfate as the globulin precipitant in the determination of proteins in blood. J. Biol. Chem., 1921, 49, 93.
18. Kunkel, H. G., The use of a serum dilution technique for the rapid and quantitative estimation of elevated globulin with special reference to liver disease. To be published.
19. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exper. Med., 1943, 78, 127.
20. Recant, L., Chargaff, E., and Hanger, F., Comparison of the cephalin-cholesterol flocculation with the thymol turbidity test. Proc. Soc. Exper. Biol. and Med., 1945, 60, 245.
21. Wolff, E. K., Buffer precipitation test (B.P.T.) for malaria. Tr. Roy. Soc. Trop. Med. & Hyg., 1939, 32, 707.
22. Kunkel, H. G., Value and limitations of the thymol turbidity test as an index of liver disease. Am. J. Med., In Press.
23. Havens, W. Paul, Jr., and Marck, R. E., A comparison of the cephalin-cholesterol flocculation and thymol turbidity tests in patients with experimentally induced infectious hepatitis. J. Clin. Invest., 1946, 25, 816.
24. Watson, C. J., and Rappaport, E. M., A comparison of the results obtained with Hanger cephalin-cholesterol flocculation test and the Maclagan thymol turbidity test in patients with liver disease. J. Lab. and Clin. Med., 1945, 30, 983.
25. Kunkel, H. G., and Hoagland, C. L., Persistence of elevated values for the thymol turbidity test following infectious hepatitis. Proc. Soc. Exper. Biol. and Med., 1946, 62, 258.
26. Tierney, N. A., and Yeomans, A., Metabolic studies in louse-borne typhus. Observations on serum electrolyte pattern, serum protein partition, and nitrogen balance. J. Clin. Invest., 1946, 25, 822.

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THE EFFECTS OF TEMPORARY CESSATION OF RENAL BLOOD FLOW IN RATS [1]

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Litten (1) observed in 1881 that temporary occlusion of the renal artery in dog and rabbit for 1½ to 2 hours was followed by tubular necrosis. In 1926 McEnery, Meyer and Ivy (2) clamped the blood vessels of both kidneys in dogs for periods of 30 minutes to 1 hour. This led to an increase in BUN [2] and some animals died in uremia. Recently, Emmel (3) described the mitochondrial and pH changes in the rat's kidney following interruption and restoration of the renal circulation. Scarff and Keele (4) removed the right kidney of rabbits and then clamped the left renal pedicle for periods up to 120 minutes. After release the BUN rose rapidly until death or reached a peak and declined with recovery. Outcome did not always depend on time of occlusion. Van Slyke *et al* (5) found that in the dog, if one kidney was removed and the renal artery of the other clamped for 3 hours, the animal would recover following an elevation of BUN. With clamping of 4 hours, only half of the dogs recovered and at 6 hours the procedure was uniformly fatal with uremic death in 4 to 8 days. Thus renal occlusion of more than 4 hours was considered irreversible. Selkurt (6) studied the effect of temporary clamping of the left renal artery of the dog on subsequent left renal clearances. Ischemia of 20 minutes' duration practically eliminated creatinine and para-amino-hippuric acid clearances during observations extending for 136 minutes after release of clamp.

In the following experiments a study was made of the effects of temporary complete renal ischemia in rats.

METHODS

Adult white male rats, weighing about 200 grams each, were anesthetized with intraperitoneal sodium pentobarbital given in dosage of 25 to 35 mgm. per kgm. of body weight (0.3 to 0.4 ml. of a 2 per cent solution). With aseptic technique the kidneys were then exposed by lumbar incisions extending through the oblique abdominal muscles, the renal pedicles isolated, and the renal artery and vein occluded by means of bulldog clamps. The latter were applied on right and left sides usually within an interval of 20 seconds. Ureters were not occluded. Except for separation of adrenal from the kidney, no dissection of peri-renal fat was made. In all rats 0.5 ml. heparin was injected into the tail vein about 10 minutes before clamping to retard intrarenal thrombosis during the period of vascular occlusion. The heparin did not cause excessive bleeding. Food and water were withheld for about 10 hours prior to anesthesia, and were given *ad libitum* following the operation.

Complete renal ischemia was maintained bilaterally for exact periods of time, *i.e.*, 30 minutes, 1 hour, 90 minutes, and 2 hours. The kidneys were generally dark purple at the end of these periods, but all became uniformly pink either within a matter of seconds or a few minutes after removal of the clamps. A group of 15 rats was studied at each time interval.

Renal function was followed by determination of BUN, made according to the method of Ormsby (7) with 0.1 or 0.2 ml. of blood from the tail. A control level was obtained prior to operation and then values were determined at fixed intervals following removal of the clamps, *i.e.*, 6 hours, 24 hours, 2, 3, and 4 days, and in some instances 6 and 8 days. A sample of heart's blood was taken for BUN in rats which died in uremia.

The kidneys of all rats were removed for gross and microscopic study, either at the time of death or when the animals were sacrificed, usually from 4 to 8 days after the period of complete renal ischemia. A few rats in the 90-minute or 2-hour group showed renal infarction at autopsy and these were discarded and replaced. There was no observable post-operative infection.

Additional experiments indicated that the method used to produce complete renal ischemia was effective. In 5 rats coronal section of the kidneys after application of the clamps was not followed by bleeding from the cut surface. Profuse hemorrhage occurred when the clamps were released. Ten rats were sacrificed after periods of complete renal ischemia ranging from 30 minutes to 2 hours. In 2 of these animals 1 kidney was left unclamped to serve as a control. Immediately after sacrifice, with the clamps still in place, the thoracic aorta was cannulated and perfused at an average pressure of 150 mm.

[1] Aided by a grant from the Elisabeth Severance Prentiss Foundation.

[2] BUN denotes blood urea nitrogen.

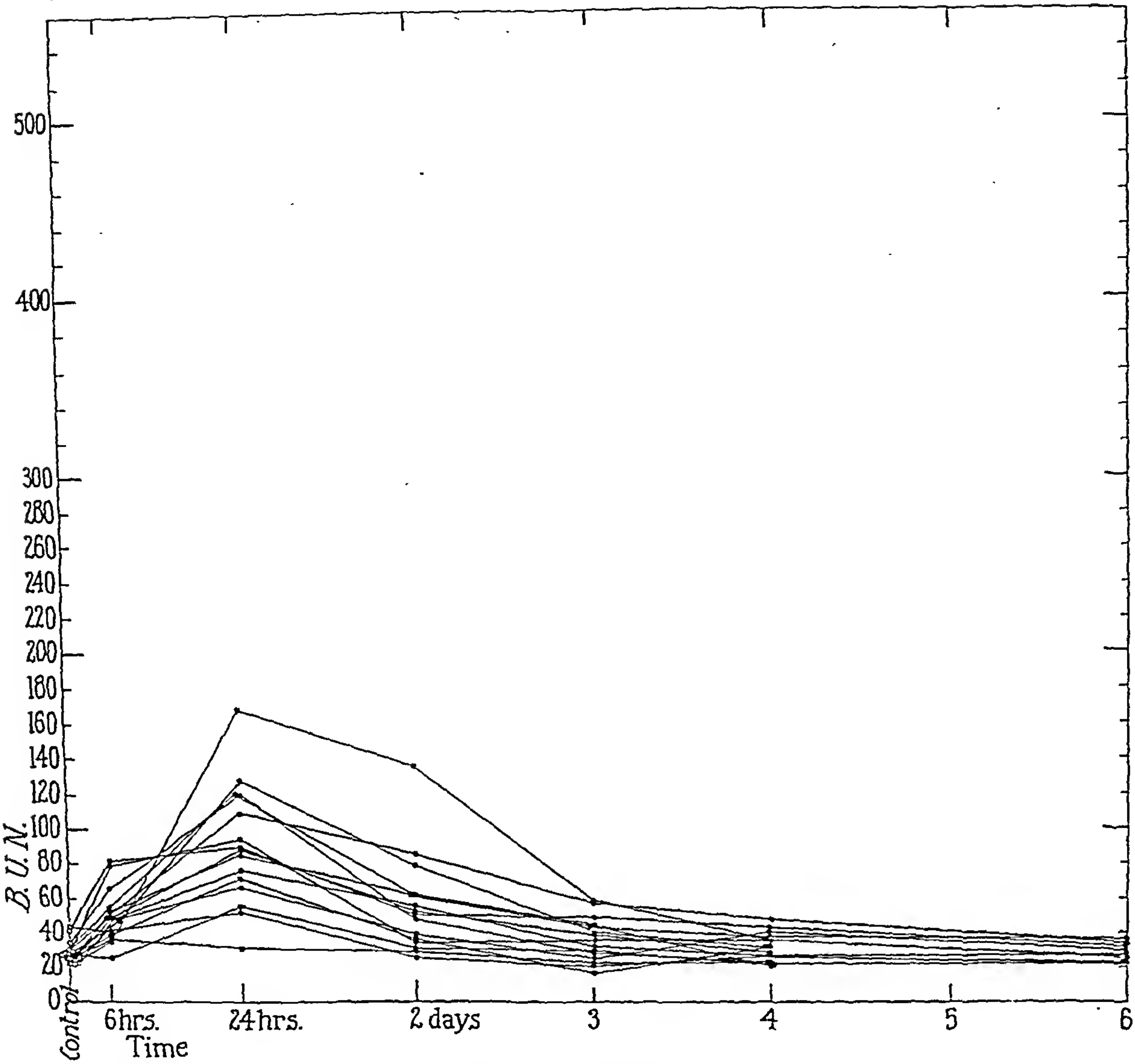

FIG. 1. BLOOD UREA NITROGEN FOLLOWING COMPLETE RENAL ISCHEMIA FOR 30 MINUTES

Hg with warm physiological saline followed by Higgin's india ink diluted 1 to 10 with saline. No gross coloration of the clamped kidneys was obtained. Microscopically, staining with india ink was practically negligible although pigment was observed occasionally in capillaries beneath pelvic mucosa and rarely in a few capillaries within the medulla.

RESULTS

After removal of the clamps, there was a progressive rise in BUN either until death or until a peak was reached, followed by a gradual decline and survival (Figures 1 to 4[3]). The animals which died showed typical manifestations of uremia, *i.e.*, weakness, apathy, somnolence, and diarrhea. The mortality among the 4 groups of rats is recorded in Table I.

[3] Values for BUN are in mgm. per 100 ml. of blood. For convenience the time of death is given to the nearest day.

TABLE I

Mortality in 60 rats following complete renal ischemia

Number of rats	Duration of complete renal ischemia	Mortality
		per cent
15	30 minutes	0
15	1 hour	13
15	90 minutes	73
15	2 hours	100

Fifteen rats with complete renal ischemia for 30 minutes. There was no mortality in this group. In 14 of the 15 rats the BUN rose to a maximum 24 hours after return of renal circulation, the values ranging from 52 to 168, with a mean of 94. In 1 rat with a control BUN of 25, a rise to only 37 was obtained at 6 hours. In every instance the BUN reverted to normal by the end of 4 or 6 days.

Fifteen rats with complete renal ischemia for 1 hour. Only 2 of the 15 rats in this group died, a mortality of 13 per cent. In the fatal cases death occurred 3 days after release of the clamps and the terminal BUN values were 312 and 400, respectively.

Thirteen rats survived the renal ischemia. The maximum rise in BUN (105 to 256) usually occurred at 1 or 2 days. By the end of 4 or 6 days the values in 6 animals had declined to normal, but were still slightly elevated in the remaining 7, *i.e.*, 40 to 82.

Fifteen rats with complete renal ischemia for 90 minutes. Eleven of the rats died in uremia, a mortality of 73 per cent. Death occurred from 2 to 6 days after the renal circulation was re-

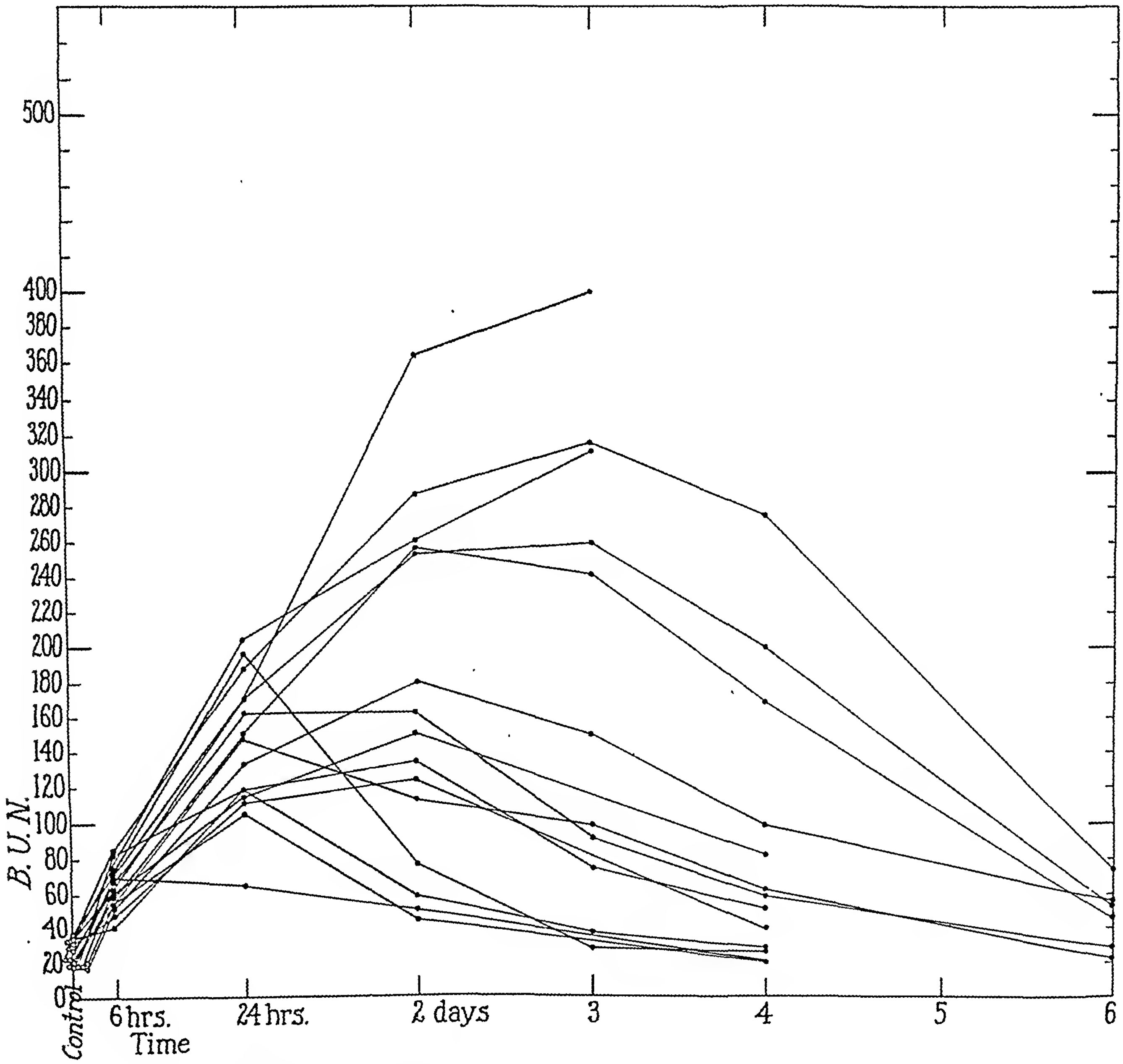

Fig. 2. Blood Urea Nitrogen following Complete Renal Ischemia for 1 Hour

ored and the terminal BUN ranged from 244 560.

Four animals in this group survived the renal chemia after attaining a BUN ranging from 0 to 260. At the time of sacrifice on the eighth y, the blood level was still slightly elevated in rats (Figure 3).

Fifteen rats with complete renal ischemia for hours. All rats in this group showed a pro-ressive rise in BUN and died in uremia from to 4 days after renewal of renal circulation Figure 4).

Morphology

The kidneys of most rats in the 30-minute group and of some in the 1-hour group showed no significant gross abnormality. In the remaining animals the kidneys were generally normal in size or slightly enlarged and frequently revealed bulging on section. The most distinctive change was the presence of a homogeneous cream-colored zone involving the entire inner portion of cortex. This contrasted with the outer cortex which was light red or brown. The medulla was pale pink and not remarkable.

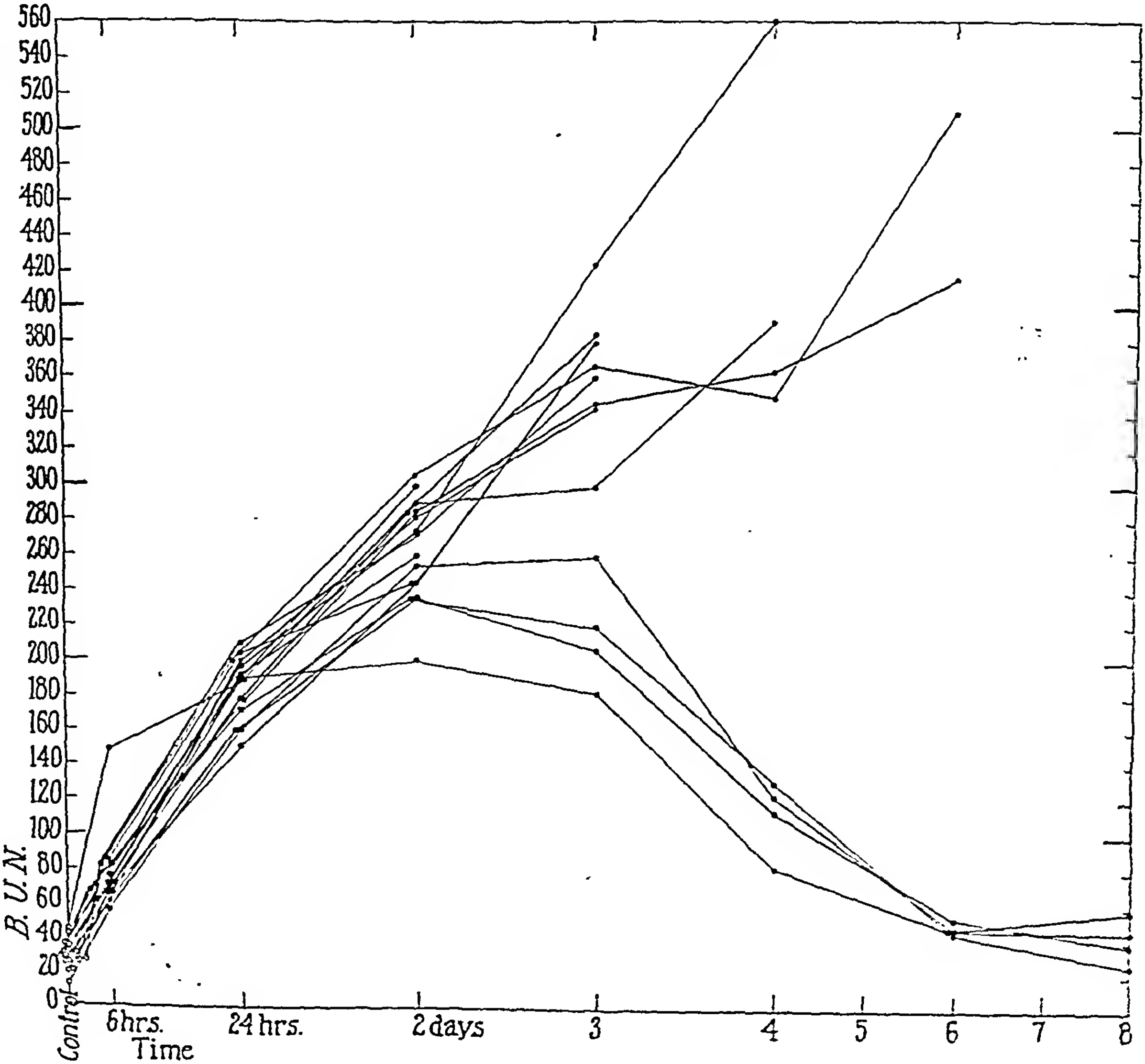

FIG. 3. BLOOD UREA NITROGEN FOLLOWING COMPLETE RENAL ISCHEMIA FOR 90 MINUTES

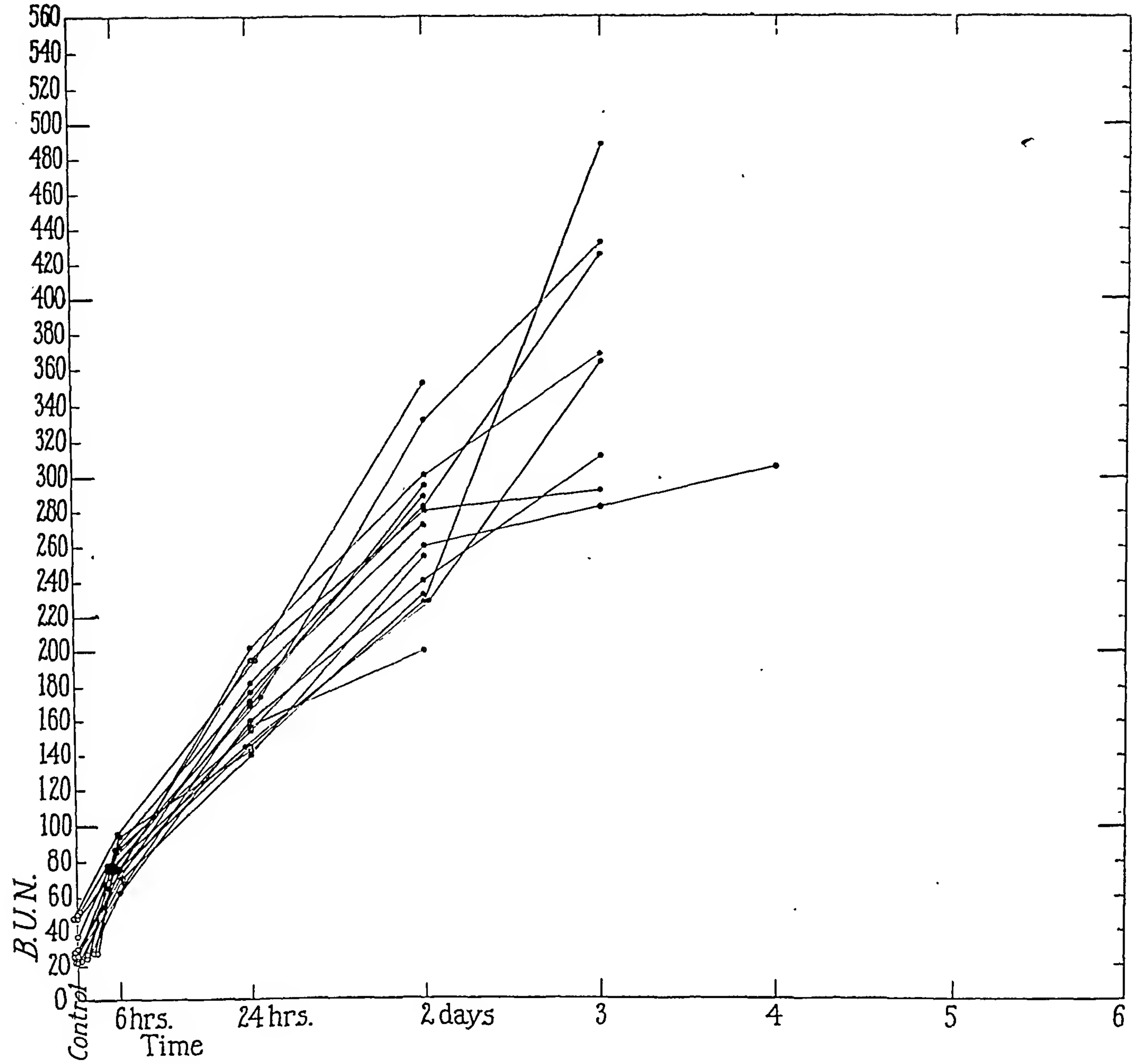

FIG. 4. BLOOD UREA NITROGEN FOLLOWING COMPLETE RENAL ISCHEMIA FOR 2 HOURS

Microscopically there was necrosis of tubules, involving principally proximal convoluted tubules in their distal or descending segments. The lesion was usually slight in rats with a 30-minute period of complete renal ischemia, more severe in degree and distribution in rats with ischemia of 1 hour's duration, and massive in animals which died in uremia, *i.e.*, chiefly those in the 90-minute and 2-hour groups.

The necrotic tubules showed loss of nuclei and the lumens were occluded by coarse acidophilic débris derived from desquamated cells. Loops of Henle and distal convoluted tubules contained acidophilic hyaline casts, often with interspersed pyknotic nuclear fragments, but the epithelium was preserved. The lumens of the collecting tubules were filled with amorphous pink staining casts. In general the glomeruli showed no significant morphologic change although some were reduced in size. No lesion of blood vessels was found. In the vicinity of necrotic tubules there was a slight focal interstitial exudate of lymphocytes and a few polymorphonuclear leucocytes.

In the more severely injured kidneys, the desquamated material within the necrotic tubules was frequently the seat of superimposed calcific de-

posit. This was negligible or absent in all rats with renal ischemia for 30 minutes and in most rats with ischemia of 1 hour's duration. The calcific material was in granular basophilic form in the early stage and later appeared as large, coarse, discrete or confluent masses attached to the lining of the tubules, often filling the lumens, and completely replacing the necrotic débris.

Regeneration of necrotic tubules was indicated by formation of new lining epithelial cells with vesicular nuclei and frequent mitoses. When sacrificed, all rats in the 30-minute group and most of those in the 1-hour group showed almost complete removal of necrotic material. The tubular lumens were generally dilated and lined by intact flat epithelium. There was marked reduction in the number of casts. Rats which died in uremia usually showed only slight or negligible repair. In 7 surviving rats, *i.e.*, 4 in the 1-hour group and 3 with 90-minute renal ischemia, regeneration was incomplete since numerous tubules contained calcific masses in place of the necrotic débris. A similar change was also observed in several rats among the 90-minute and 2-hour groups which died in uremia.

DISCUSSION

Rats readily survived a 30-minute period of complete renal ischemia and usually survived a 1-hour period. The mortality was high (73 per cent) at 90 minutes and reached 100 per cent at 2 hours. Animals which died showed progressive elevation of BUN and death occurred in uremia from 2 to 6 days after renal blood flow was restored. In rats which survived, the BUN rose to a maximum from 6 hours to 3 days after release of the constricting clamps, depending on duration of the ischemia, and then returned to normal or slightly elevated values within the next few days.

Only 2 hours of complete renal ischemia were required to produce death consistently as compared to 4 hours or more in the dogs studied by Van Slyke *et al* (5). Presumably this is due to a difference in species. Difference in experimental procedure, *i.e.*, simultaneous bilateral occlusion of renal vessels as against Van Slyke's technique of resecting one kidney and clamping the renal artery of the other, is evidently not significant. Following resection of the right kidney, we clamped the left renal artery and vein in 6 rats and the left renal artery alone in 6 additional rats. All 12 animals died in uremia from 1 to 3 days after a 2-hour period of vascular occlusion.

In our experiments, cessation of blood flow to the kidneys was produced by clamps which completely occluded main renal artery and vein. During the period of occlusion there was apparently no significant collateral circulation to the kidneys. Release of clamps was followed by almost immediate return of circulation and the subsequent presence of intact blood supply to the kidneys was confirmed morphologically by the absence of thrombosis or infarction. In all animals the renal lesion was uniform in both kidneys.

There was fairly good correlation between duration of renal ischemia and the resulting morphological injury to the kidneys after renewal of blood flow. With the longer periods of vascular occlusion, tubular necrosis of the kidneys was usually more diffuse and wider in distribution in the individual nephrons. Also, in rats which survived, the mean rise in BUN was more pronounced and sustained and was followed by a slower decline.

The principal change was necrosis of proximal convoluted tubules, especially the distal or descending segments. The reason for this localization is not known. The lumens of the tubules were blocked by coarse anuclear acidophilic masses, representing necrotic desquamated epithelial cells. Significant damage to epithelium of loops of Henle, to distal convoluted and collecting tubules, as well as to glomeruli, was not morphologically evident.

These observations are in contrast with the kidneys of human crush syndrome (8) or the experimental lesion in the rat (9), which show lower nephron nephrosis with involvement of the ascending loops of Henle and the distal convoluted tubules. The latter are often necrotic whereas the proximal tubules reveal only moderate degenerative change in the nature of cloudy swelling. The lesion also differs from that of rats with fatal tourniquet shock in which there is degenerative change, mainly of the proximal convoluted tubules, but no necrosis and only slight

cast formation (10). However, because of the short survival time of such rats, from a few to several hours, the renal lesions are not strictly comparable with those due to complete ischemia.

In this study the damage to the kidneys was due primarily to the temporary period of complete anoxia during which the cells presumably underwent profound chemical alteration. However, return of circulation was required to bring about the development of cellular necrosis.

In rats with a 30-minute period of complete ischemia, the renal lesion was relatively slight and was followed by complete regeneration. This was also true of most rats in the 1-hour group even though kidney damage was more extensive. After 4 to 8 days the tubules were cleared of necrotic material and presented dilated lumens lined by newly formed flat epithelium. However, in 4 animals of the 1-hour group and in 3 surviving rats with 90-minute renal ischemia, even though the majority of tubules were effectively restored, some revealed failure of regeneration associated with massive calcific deposit. Such tubular injury perhaps explains the failure of the BUN in these animals to return to normal at the time of sacrifice. Most rats which died in uremia showed only slight or negligible regeneration. This does not necessarily indicate an intrinsically irreversible lesion since death occurred before repair could be effective.

SUMMARY

1. Rats readily survived a 30-minute period of complete renal ischemia and usually survived a 1-hour period. The mortality at 90 minutes was 73 per cent and at 2 hours the procedure was uniformly fatal.

2. After circulation was restored, the blood urea nitrogen rose rapidly until death, or reached a maximum level and then declined with survival of the animal.

3. The principal morphologic change was necrosis of the descending segments of the proximal convoluted tubules.

4. The renal lesion was not similar to that observed in human or experimental crush syndrome or in rats with fatal tourniquet shock.

BIBLIOGRAPHY

1. Litten, M., Ueber pathologische Verkalkungen und Kalkmetastasen in den Nieren. Virchows Arch., 1881, 83, 508.
2. McEnery, E. T., Meyer, J., and Ivy, A. C., Studies on nephritis; physiologic and anatomic changes following temporary ischemia of the kidneys. J. Lab. & Clin. Med., 1927, 12, 349.
3. Emmel, V. M., Mitochondrial and pH changes in the rat's kidney following interruption and restoration of the renal circulation. Anat. Rec., 1940, 78, 361.
4. Scarff, R. W., and Keele, C. A., The effects of temporary occlusion of the renal circulation in the rabbit. Brit. J. Exper. Path., 1943, 24, 147.
5. Van Slyke, D. D., Phillips, R. A., Hamilton, P. B., Archibald, R. M., Dole, V. P., and Emerson, K., Effect of shock on the kidney. Tr. A. Am. Physicians, 1944, 58, 119.
6. Selkurt, E. E., The changes in renal clearance following complete ischemia of the kidney. Am. J. Physiol., 1945, 144, 395.
7. Ormsby, A. A., Direct colorimetric method for determination of urea in blood and urine. J. Biol. Chem., 1942., 146, 595.
8. Lueké, B., Lower nephron nephrosis. (The renal lesions of the crush syndrome, of burns, transfusions, and other conditions affecting the lower segments of the nephrons.) The Military Surgeon, 1946, 99, 371.
9. Corcoran, A. C., and Page, J. H., Post-traumatic renal injury; summary of experimental observations. Arch. Surg., 1945, 51, 93.
10. Unpublished observations of the authors.

SEROLOGICAL RESPONSE TO INTRANASAL ADMINISTRATION OF INACTIVE INFLUENZA VIRUS IN CHILDREN [1]

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The published reports on antibody response of human individuals to the inhalation of influenza virus have been most concerned with the effect of active or "attenuated" virus (1 to 9). In some instances experimental disease was induced to test the efficacy of previous vaccination or to note the effects of inhalation of active virus on the production of antibodies against influenza. Burnet (7) reported that 68 to 80 per cent of a group he studied did not show significant changes in antibody titer when they inhaled an "attenuated" influenza virus preparation. It has been demonstrated (6 to 9) that inhalation of active influenza virus can give a modified form of the disease and result in good antibody responses. It has also been shown (1c, 2 to 5) that subcutaneously injected influenza vaccine results in protection against experimentally induced disease. Recently Henle, Henle, Stokes and Maris (10), using serologically determined antibody response as a basis for comparison, concluded that inhalation of active or inactive virus, mostly unconcentrated, was not as effective as subcutaneously injected vaccine.

The present study was conducted to determine the antigenic effect of inactive virus in concentrated form given intranasally, by a single or multiple spray. This effect was compared to that obtained with subcutaneous vaccination.

MATERIAL AND METHODS

Virus preparation

The inactive virus preparation was prepared by a commercial biological laboratory from infected allantoic fluid, centrifuged 2 times in a Sharples centrifuge and furnished to the Influenza Commission by W. M. Stanley. The virus was inactivated with formalin in a final concentration of 1:2,000, and phenyl mercuric nitrate to a concentration of 1:100,000 was added. The material originally comprised equal parts of Type A influenza virus (½ PR8 strain [11] and ½ Weiss strain [12]) and the Lee strain (13) of Type B influenza virus. It was estimated to contain 10 mgm. of protein per ml. and was received in this laboratory on September 11, 1944. The material was diluted just prior to use on September 30, 1945. When tested serologically on March 13, 1946, a 1:5 dilution in physiological saline gave a hemagglutinating titer of 51,200 and a 1:40 dilution gave a titer of 5,120 (14).

Subjects

The children were from 3 cottages of 50 boys each at the Wayne County Training School, Northville, Michigan. The range in age was from 8 to 14 years. Most of the children used were smaller than average in size and of lower than average mental level. The only selection made within the group was the elimination of any children with allergic tendencies.

A total of 80 children was divided into 4 groups. Twenty children from Cottage 1 were used in Group I; 20 children from Cottage 9 constituted Group II; and 20 children from Cottage 6 made up Group III. The fourth group contained 10 children from Cottage 1 and 10 children from Cottage 9. In the course of the study one child was lost from each group except Group I.

In each cottage there were children who were unvaccinated and who did not receive any control fluid. In Cottage 1 this group consisted of 20 children; in Cottage 9, 20 children; and in Cottage 6, 30 children.

In the vaccinated group there were 11 children who had received a combined influenza virus, Types A and B, vaccine in January, 1944. Five of them were in Group I; 3 in Group II; and 3 in Group III. None of the children in the control group had been given any previous vaccination against influenza viruses.

Administration of inactive virus preparation

A 1:5 dilution in physiological salt solution was employed for the intranasal sprays. The atomizer was obtained from the Vaponephrin Company, Upper Darby, Pennsylvania; with 10 lbs. pressure of nitrogen gas it delivered approximately 0.25 ml. per 2 minutes. Each individual was sprayed for 1 minute in each nostril. The adapter was washed with antiseptic solution before use

[1] This study was aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

TABLE I.

Mean antibody titers for the four groups

Strain	Group I (5 sprays) (20 children)				Group II (1 spray) (19 children)				Group III (subcutaneous) (19 children)				Group IV (inhalation control) (19 children)		
	initial	*2 wks.*	*fold increase*	*5 mos.*	*initial*	*2 wks.*	*fold increase*	*5 mos.*	*initial*	*2 wks.*	*fold increase*	*5 mos.*	*initial*	*2 wks.*	*5 mos.*
PR8	224	745	3×	563	148	443	3×	297	310	1363	4×	701	134	141	128
Weiss	192	588	3×	499	218	538	2×	148	269	942	3×	499	196	196	196
Lee	58	691	12×	435	40	294	7×	430	84	1935	23×	891	32	32	99.8

by another subject. Precautions were taken to minimize the inhalation of the sprayed material by any other than the selected subjects. Each child was brought individually into the spray room which was located on the upper floor of Cottage 1. The room was unused except for spraying throughout the study period.

For subcutaneous administration 1.0 ml. of a 1:40 dilution of the inactive virus was given in the left arm. A clean sterile needle was used for each individual.

Group I received intranasal inhalations of the 1:5 dilution of inactive virus on October 1, 2, 3, 4, and 5, 1945. Group II received 1 intranasal inhalation of the 1:5 dilution on October 2, 1945. Group III was given 1.0 ml. of the 1:40 dilution subcutaneously on October 3, 1945. Group IV received 1 intranasal inhalation of sterile normal allantoic fluid on October 1, 1945. These last inhalations were given in a cottage well removed from that in which the spraying of the inactive virus preparation was carried out.

Oral temperatures were taken on October 1, 2, 3, 4, and 5, 1945, in Groups I, II and IV. Signs and symptoms of reactions were recorded daily in all groups. Group III had oral temperatures recorded on October 3, 4, and 5; on the latter 2 days the site of subcutaneous inoculation was examined and the children observed for systemic signs and symptoms.

Serological studies

Blood was drawn from each individual immediately before vaccination, 2 weeks after vaccination, and 5 months after vaccination. The second blood specimens were obtained from those receiving 5 intranasal inhalations 2 weeks after the third dose. Agglutination-inhibition titrations of chicken red blood cells were done according to Salk's modification (14) of Hirst's method (15). The sera from a given individual were all tested at the same time. Agglutination-inhibition was measured against the PR8 and Weiss strains of Type A virus, and the Lee strain of Type B virus. The same virus preparations were used for all tests. The chicken red blood cells were taken from animals whose cells had been checked repeatedly and found to be satisfactory.

RESULTS

Response to influenza virus, Type A

The sera from the 4 study groups exhibited varying degrees of agglutination-inhibition with the PR8 strain of Type A influenza virus. A comparison of the serological findings in the 3 vaccinated groups and the control group is presented in Figures 1, 2 and 3. The modified arithmetic means are summarized for all groups in Table I.[2]

The 20 individuals in *Group I,* who received 5 intranasal inhalations, had an initial mean titer against the PR8 strain of 224; 2 weeks later this rose to a mean of 745 and represented an increase of 3.3 times the initial value. Five months later it had dropped to 563. The distribution of these sera indicates that 2 weeks after vaccination, 12 had a 4-fold or greater increase in titer against

TABLE II

Comparison of antibody level before and 2 weeks after administration of inactive virus

Strain	Group I (20 children)		Group II (19 children)		Group III (19 children)	
	2× or less	*4× or more*	*2× or less*	*4× or more*	*2× or less*	*4× or more*
PR8	8*	12†	12	7	5	14
Weiss	11	9	14	5	10	9
Lee	4	16	4	15	1	18

* Number of individuals with 2-fold or less increase in titers.

† Number of individuals with 4-fold or greater increase in titers.

[2] All titers are expressed as the reciprocal of the highest final dilution of serum giving inhibition of agglutination. The mean titration figures for each group of sera were arrived at in the following manner. The numbers 1, 2, 3, etc., were assigned to the dilutions of sera (*e.g.,* $1 = <32$; $2 = 32$; $3 = 64$; *et seq.*). The number of individuals having a certain titer was then multiplied by the number assigned to that titer (*e.g.,* $32 = 2$; therefore $2 \times 3 = 6$). The totals for each designated number were then added and this result divided by the number of sera in the group. This was then reconverted to the titer it represented and any fraction was multipled by the titer the whole number represented (*e.g.,* 2.35 is equivalent to $32 + [32 \times 0.35]$).

the PR8 strain, and 8 had a 2-fold increase or less, when compared to the titers before the virus preparation was administered (Table II).

The sera from the 19 children in *Group II* when measured against the PR8 strain gave an initial mean titer of 148 with a rise to 443, an increase of approximately 3 times the initial titer, 2 weeks after the single inhaled dose. As indicated in Table II, 7 sera had 4-fold or greater increases in titer and 12 had 2-fold or less. The mean value 5 months later had dropped to 297.

When titrated with the PR8 strain, the sera of the 19 subcutaneously vaccinated children in *Group III* had an initial mean titer of 310; 2 weeks after vaccination, 1,363; and 5 months later, 701. Of the 19 sera tested, 14 had a 4-fold or greater increase in titer 2 weeks after vaccination, and 5 had a 2-fold increase or less. The 5

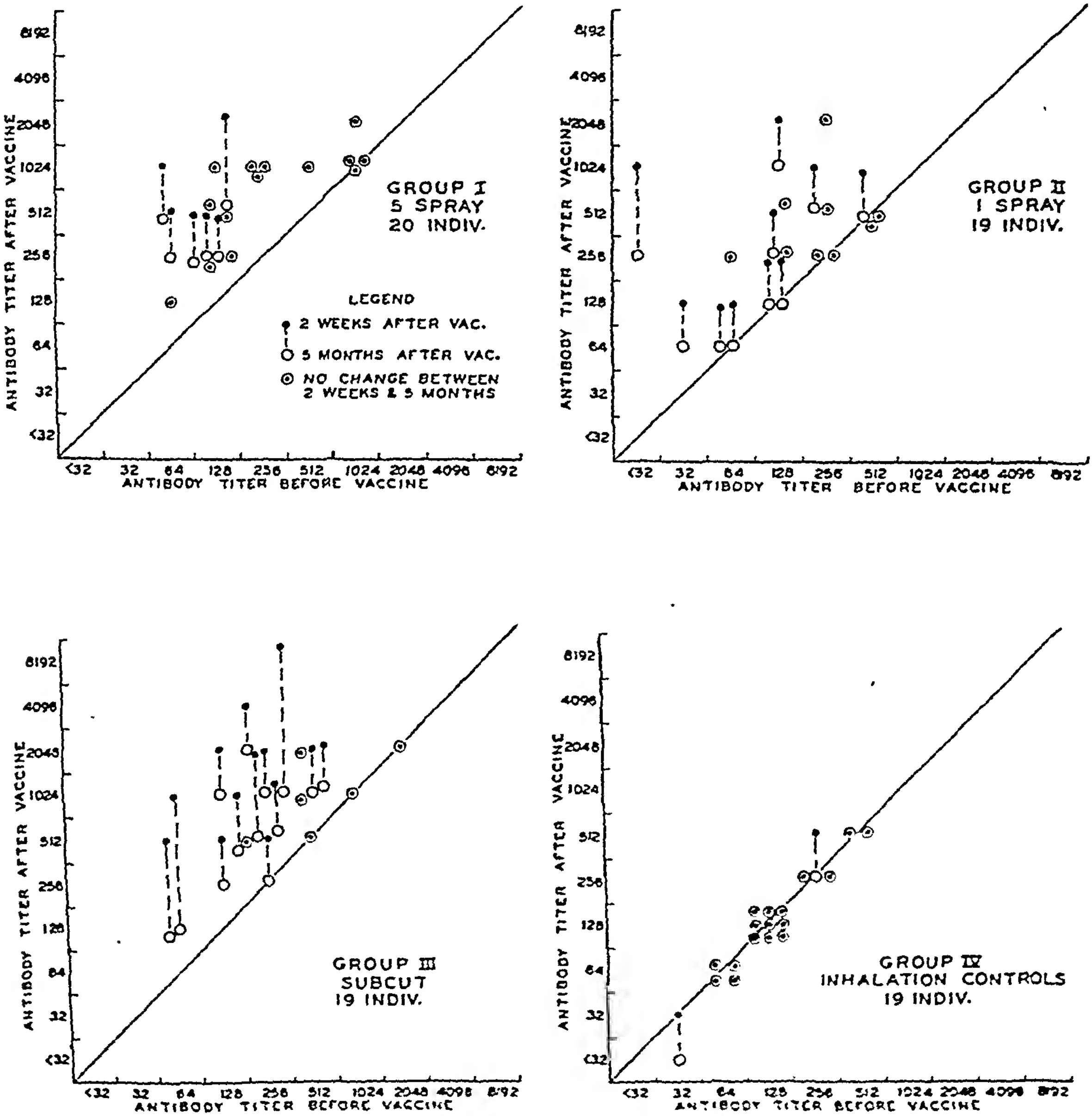

FIG. 1. CHANGE IN ANTIBODY TITER MEASURED AGAINST THE PR8 STRAIN 2 WEEKS AND 5 MONTHS AFTER VACCINE WAS ADMINISTERED

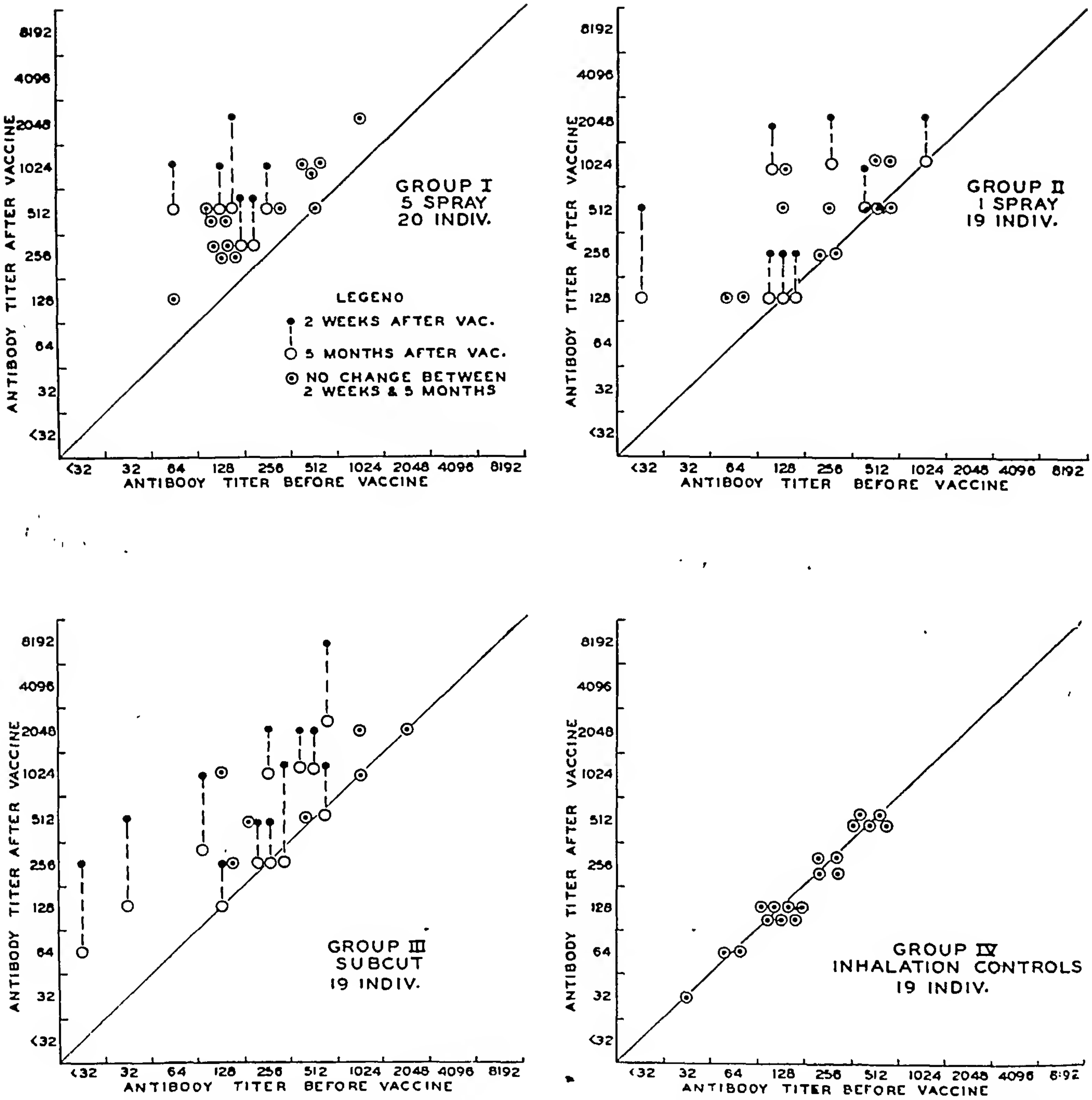

FIG. 2. CHANGE IN ANTIBODY TITER MEASURED AGAINST THE WEISS STRAIN 2 WEEKS AND 5 MONTHS AFTER VACCINE WAS ADMINISTERED

who showed the increases of 2-fold or less all had initial titers of 256 or higher. Five months after vaccination the sera showed a sharp drop with 9 falling 2-fold, 1 falling 4-fold, 2 falling 8-fold; and 6 had no change in titer (Figure 1).

The subjects who received the single inhalation of normal allantoic fluid in *Group IV* showed no appreciable change in titers when measured against the PR8 strain of influenza virus. However, it is worth noting that the sera of 2 individuals showing changes in titer were decreases against the PR8 strain; and later it will be shown that the control group when measured against the Lee strain of Type B virus showed marked rises in titer.

The serological responses of the 4 groups were similar to the above patterns when measured against the Weiss strain of Type A influenza virus (Figure 2). The differences are most noticeable in Group III, where the PR8 titers were

greater than the Weiss titers, and 5 months after vaccination less of a drop occurred with the former. The difficulty in interpreting antibody response to various strains of influenza virus has been commented on by others. Burnet and Lush (16) have assumed that any measurement of specific antibody will always include some non-specific antibody. This was shown to be true by Hirst (17). More recent evidence lends weight to the supposition that the agglutination-inhibition mechanism is a complex one and that too exact interpretation of results may lead to erroneus conclusions (18). In any event, a study of Table I and the spot charts does show a consistent picture of high initial antibody titers to both strains of Type A virus. Whether this represents previous specific experience on the part of the subjects with these Type A virus strains or is related to peculiarities of the PR8 and Weiss preparations, to non-specific characteristics of the sera used in the tests, or to combinations of these influences, one cannot surely state. Certainly, in view of the Type A epidemic in 1943 (12), the

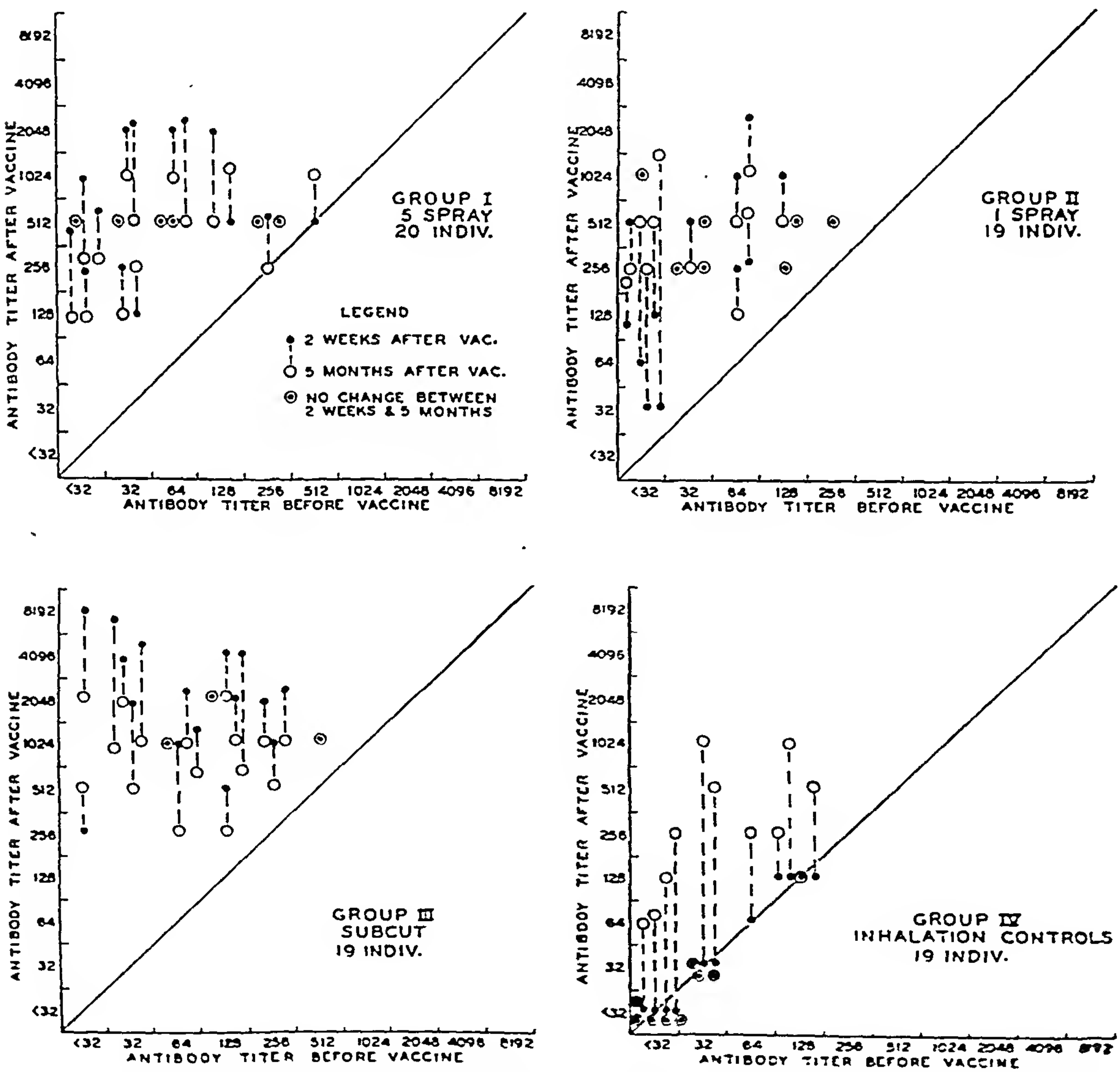

FIG. 3. CHANGE IN ANTIBODY TITER MEASURED AGAINST THE LEE STRAIN 2 WEEKS AND 5 MONTHS AFTER VACCINE WAS ADMINISTERED

possibility of previous contact with A strains is a strong one.

Responses to the Lee strain of Type B influenza virus

When the 4 groups of sera were tested against the Lee strain of Type B virus, a marked difference in antibody response, when compared with A, was apparent (Figure 3). The mean values listed in Table I show much lower initial titers for all 3 groups against the B strain. Possibly this is an indication of less experience with the Type B strains, resulting in a fuller response to the antigenic stimulus.

In *Group I,* after 5 daily sprays, the initial mean value was 58; this rose to 691 2 weeks after vaccination, representing an increase of 12 times the initial titer. Five months later the mean was 435. Table II lists the distribution when the sera taken 2 weeks after vaccination were compared with the initial values. Sixteen of the 20 in the group had a 4-fold or greater increase in titer and 4 showed a 2-fold increase. The sera taken 5 months after vaccination did not show the same drop in titers that was noted with the A strains. Although 11 sera did have decreases in titer, 3 sera had 2-fold rises in titer. In the interval between the bloods drawn 2 weeks and 5 months after vaccination, an epidemic of influenza B was noted. This will be discussed in more detail later.

The *Group II* sera, from the individuals receiving the single spray of inactive virus preparation, had similar responses when measured against the Lee strain of Type B influenza virus. The mean titers for the 3 sets of sera in this group were: initial, 40; 2 weeks after vaccination, 294; 5 months after vaccination, 430. The sera taken 2 weeks after vaccination, when compared to the pre-vaccination sera, showed 15 with a 4-fold or greater increase in titer and 4 with a 2-fold increase. A comparison with the sera taken 5 months later gave 6 with a drop in titer and an equal number with 2-fold or greater increase in titer. The spot charts in Figure 3 show this change. The same situation existed as in Group I; namely, an epidemic of Type B influenza that involved enough members of this group to cause a definite increase in a number of the agglutination-inhibition titers against the Lee strain of Type B influenza virus.

Group III, after the subcutaneous dose, showed the most striking responses against the Lee strain. The mean titers were: initial, 84; 2 weeks after vaccination, 1,935; 5 months after vaccination, 891. The increase 2 weeks after vaccination was approximately 23 times the initial mean titer and 5 months later it was 10 times the initial titer. The distribution of the 19 sera in this group is shown in Table II and indicates that 18 of the bloods taken 2 weeks after vaccination exhibited 4-fold or greater responses and one had a 2-fold increase when compared to the pre-vaccination levels. Of the sera taken 5 months after vaccination 9 had 2-fold decreases in titer and 6 had 4-fold or greater decreases in titer. In this same set of sera there were 3 with no change in titer and 1 serum with a 2-fold increase in titer.

Group IV, the sera from the inhalation controls, when titrated against the Lee strain of Type B influenza virus, showed no change between the initial titers and those of 2 weeks later. It has been previously stated that an outbreak of Type B influenza occurred in the institution in the interval between the 2-week bleedings and the 5-month bleedings. During this period the members of the various groups were under close clinical observation. When the 19 sera taken 5 months after vaccination were titrated against the Lee strain, 10 had increases in titer ranging from 2-fold to 32-fold (Figure 3). All but 1 of the 10 were 4-fold or greater increases in titer.

The levels of antibody which will be attained by human individuals in response to influenza vaccination may vary widely. Results of small groups of subjects cannot be too freely evaluated. The above data can only indicate broad, general trends of antibody response. Even the fact that the response to the strain of Type B virus was numerically better than A must be accepted with reservation. Other studies have indicated that the Lee strain, measured in terms of protection against natural and experimental disease, induces a stauncher degree of protection than certain A strains of influenza virus (4, 5, 19). The low initial titers to the Lee strain were associated with more striking increases in titer 2 weeks after vaccination and, conversely, the higher original

titers to the A strains minimized the increments in titer after vaccination. The figures listed in Table I indicate that the mean-fold increase was greater against the Lee strain than either PR8 or Weiss. However, the mean titers of the 2-week bleedings were not so sharply differentiated except in Group III. If generalizations are to be made one can point out that the mean-fold increase in titer to B was better than that to A although the differences between titers were not consistent; that subcutaneous vaccination appeared to be more effective than intranasal although rather uniform responses were obtained with the latter. It is of interest to note that the group receiving the 5 sprays had a total of 1.25 ml. of a 1:5 dilution of vaccine whereas the subcutaneous group received 1 ml. of the 1:40 dilution of vaccine, strongly suggesting that subcutaneously injected vaccine was more efficiently utilized. These studies with concentrated inactive material support the impression obtained from the results of Henle *et al* (10).

The subcutaneous vaccinations resulted in excellent antibody production against the Lee strain but in a lesser response to the A strains. Numerically the antibody titers in the subcutaneous group were higher than in previous experiments with adults (3, 5, 19). Proof of whether this represents chance or that children in this age group do respond better and more consistently, would require studies with large groups of children.

As already mentioned, some of the subjects in the vaccinated groups had received a combined A and B influenza virus vaccine in January, 1944. Their initial anti-A titers were not significantly different from the remainder of the groups so they could not be considered the cause of the higher initial titers to Type A. For the Lee strain the initial titers of those who received vaccine in 1944 were 2 times greater than the rest of the group. However, the number within each group was small enough to show only a minimal influence on the height of the mean initial titer for each study group.

Reactions

The reactions to the intranasal inhalation of the combined inactivated viruses were minimal. In the group who received the 5 daily sprays, 6 children had oral temperatures between 100.0° and 100.8° F. on the third day. Two children, after the first spray, complained of a slight sensation of fullness in the head. Two children on the fourth day had temperatures between 100.0° and 100.2° F. The single inhalation group and the control inhalation group had no reactions. Twelve of the children in Group III complained of local reactions 24 hours after the subcutaneous inoculation. Of the 12 children, 3 had slight erythema; 7 had tenderness, heat and erythema; and 2 had tenderness, heat, redness and swelling at the site of injection with axillary nodes palpable on the affected side. There were 3 children in this group with temperatures between 100.4° and 100.8° F.; and 1 child, who had a severe local reaction, complained of dizziness and slight headache.

CLINICAL OBSERVATIONS

From December, 1945, through January, 1946

Following the vaccinations done in October, 1945, periodic observations were made to determine influenza virus infection in the institution. On November 10, 1945, Type B influenza virus was isolated in Michigan (19) and on December 13, 1945, several children at the Training School reported to the sick ward with an acute illness characterized by fever, chills, prostration, headache and sore throat. The number of case throughout the school reached a peak on Dece ber 18 and fell off gradually until only occasi cases were occurring from December 18 th January 30, 1946. From throat washing of the children admitted to the sick December 18, Type B influenza virus w ered. The children from the 3 co housed the 4 groups were followed c the interval of time covering the ou of the 58 children in the 3 va developed clinical evidence of d nine of the 89 unvaccinated 3 cottages developed clinical e This includes 4 children fro trol group.

From 69[2] cases the cli

[2] This number of indivi other cottages than those

their frequency were: sore throat, temperature elevation, rhinorrhea, headache, chills and cough. Less prominent findings were excessive lacrimation (frequently associated with the complaint of burning eyes), malaise and sneezing. The sick children showed few abnormal physical findings. Prostration and lethargy during the first 12 to 24 hours was common. Moderate injection of the pharynx and a thin watery discharge appearing on the second day and rarely becoming purulent was often noted. Prompt recovery was the rule. Two children with perforated ear drums and associated chronic otitis media developed purulent aural discharges following the primary infection. They were the only complicated cases.

The total number of cases involving the children in the 4 study groups was 12: 4 of these were from the control group; 4 were from the group receiving the single inhalation; 3 were from the group receiving the 5 daily inhalations; and 1 was from the subcutaneously vaccinated group. However, when the sera from the above groups were tested 5 months after receiving the inactive virus preparation there were some individuals who showed rises in titer above the 2-week level without any clinical evidence of disease; other children who did have clinical evidence of influenza-like disease showed no rise in antibody titer in convalescence or again 5 months after the vaccination study was started. This is most noticeable in Group IV, the 19 inhalation controls, where the antibody response to infection was not masked by the effect of the inactive virus preparation. Only 1 of the 4 subjects in this group who developed clinical signs of influenza-like disease had a rise in titer against the Lee strain. The other 3 children had no rise in titer and in every determination the titers of these 3 were 32 or under when measured against the Lee strain.[4] There were no changes in the titers of the A strains in these sera. In this same group of 19 children the titers of the sera taken 5 months after vaccination showed 10 individuals with rises in titer, one of them being the child described above. The other 9 had no record of illness. The phenomenon of sub-clinical infection, as exemplified by the 9 individuals without evidence of infection and yet having rises in antibody titer in the sera taken 5 months after vaccination, is well recognized. However, the 3 individuals with evidence of disease but without rises in agglutination-inhibition antibodies are more difficult to evaluate. The use of antibody response, as currently measured, is not feasible for the diagnosis of influenza in vaccinated populations; with such a premise, then, clinical findings must be accepted for unvaccinated subjects as well. There did not seem to be any correlation between initial titer and sub-clinical infection (20).

It can be seen from Figure 3 that some rises in titer occurred during the same interval in the other groups. The subcutaneously injected group had 1 subject with a 2-fold rise in titer who showed no evidence of disease and 1 subject, whose titer was 2048, did show clinical evidence of influenza. The 5-spray group had 3 children with 2-fold rises in titer; only 1 showed evidence of disease and 2 others in this group with influenza did not have rises in titer; however, their titers were 256 and 1,024. The single inhalation group had 6 subjects with rises in titer; 2 with 2-fold rise; 1 with 4-fold rise; 2 with 8-fold rise; and 1 with 32-fold rise. Of the 6 there were 4 with clinical evidence of disease. Such figures further indicate the difficulty in interpreting clinical disease in terms of antibody response when vaccinations are carried out. Obviously, the use of the serological test is of little help. The possibility that the rises in titer seen in sera 5 months after vaccination were a continuation of the effect of vaccination is quite remote. There were no rises in titer against the A strains. Also, enough of the children who became ill had additional sera taken during the period between the 2-week and 5-month bleedings to indicate that there was a fairly consistent drop in antibody titer associated with the vaccinations. In most instances the titers 2 weeks after illness were higher and then fell to lower levels 5 months after the inactive virus preparation was administered. No significant evaluation of the effects of administration of the virus materials upon the incidence of infection with influenza virus can be made from these observations nor was the study intended for that purpose. Nevertheless, a difference in the frequency of respiratory illness among the vaccinated and unvaccinated groups was seen.

[4] These sera were tested against a strain of Type B virus isolated during the epidemic. The results showed no rise in antibodies against the current strain.

The presence of clinically undetected influenza B infection was demonstrated by the surprisingly large number of antibody rises to Type B virus in sera obtained 5 months after vaccination while A titers were continuing to fall.

SUMMARY

The data reported here indicate that a single intranasal inhalation of an inactivated influenza virus preparation resulted in good antibody responses and that the use of 5 daily sprays gave slightly higher levels of antibody. A comparison of the effect of the 2 different doses of intranasal vaccine on the sera taken 5 months after vaccination suggests that the larger dose gave a more persistent elevation in antibody titer.

The vaccine given intranasally on 5 successive days contained approximately 8 times as much inactive virus preparation as the subcutaneous material. However, a comparison of the antibody responses indicates that subcutaneously administered vaccine gives higher and more persistent levels with smaller doses. Clinical observations were made during an epidemic of influenza B in the institution.

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BIBLIOGRAPHY

1. a. Smorodintseff, A. A., Tushinsky, M. D., Drobyshevskaya, A. I., Korovin, A .A., and Osetroff, A. I., Investigation on volunteers infected with the influenza virus. Am. J. M. Sc., 1938, 194, 159.
   b. Burnet, F. M., and Foley, M., The results of intranasal inoculation of modified and unmodified influenza virus strains in human volunteers. M. J. Australia, 1940, 2, 655.
   c. Henle, W., Henle, G., and Stokes, J., Jr., Demonstration of the efficacy of vaccination against influenza type A by experimental infection of human beings. J. Immunol., 1943, 46, 163.
   d. Francis, T., Jr., Intranasal inoculation of human inviduals with virus of epidemic influenza. Proc. Soc. Exp. Biol. & Med., 1940, 43, 337.
2. Francis, T., Jr., Salk, J. E., Pearson, H. E., and Brown, P. N., Protective effect of vaccination against induced influenza A. Preliminary Report, Proc. Soc. Exper. Biol. & Med., 1944, 55, 104.
3. Francis, T., Jr., Salk, J. E., Pearson, H. E., and Brown, P. N., Protective effect of vaccination against induced influenza A. J. Clin. Invest., 1945, 24, 536.
4. Salk, J. E., Pearson, H. E., Brown, P. N., and Francis, T., Jr., Protective effect of vaccination against induced influenza B. Preliminary Report. Proc. Soc. Exper. Biol. & Med., 1944, 55, 106.
5. Salk, J. E., Pearson, H. E., Brown, P. N., and Francis, T., Jr., Protective effect of vaccination against induced influenza B. J. Clin. Invest., 1945, 24, 547.
6. Burnet, F. M., Influenza virus B. II. Immunization of human volunteers with living attenuated virus. M. J. Australia, 1942, 1, 673.
7. Burnet, F. M., Immunization against influenza with living attenuated virus. M. J. Australia, 1943, 1, 385.
8. Mawson, J., and Swan, C., Intranasal vaccination of humans with living attenuated influenza virus strains. M. J. Australia, 1943, 1, 394.
9. Francis, T., Jr., Pearson, H. E., Salk, J. E., and Brown, P. N., Immunity in subjects artificially infected with influenza virus, Type B. Amer. J. Pub. Health, 1944, 34, 317.
10. Henle, W., Henle, G., Stokes, J., Jr., and Maris, E. P., Experimental exposure of human subjects to viruses of influenza. J. Immunol., 1946, 52, 145.
11. Francis, T., Jr., Transmission of influenza by a filtrable virus. Science, 1934, 80, 457.
12. Salk, J. E., Menke, W. J., and Francis, T., Jr., Identification of influenza virus Type A in a current outbreak of respiratory disease, J. A. M. A., 1944, 124, 93.
13. Francis, T., Jr., A new type of virus from epidemic influenza. Science, 1940, 92, 405.
14. Salk, J. E., A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. J. Immunol., 1944, 49, 87.
15. Hirst, G. K., The quantitative determination of influenza virus and antibodies by means of red cell agglutination. J. Exper. Med., 1942, 75, 49.
16. Burnet, F. M., and Lush, D., Influenza virus strains isolated from Melbourne 1939 epidemic. Australian J. Exper. Biol. and M. Sc., 1940, 18, 49.
17. Hirst, G. K., Studies of antigenic differences among strains of influenza A by means of red cell agglutination. J. Exper. Med., 1943, 78, 407.
18. Francis, T., Jr., Dissociation of hemagglutinating and antibody measuring capacities of influenza virus. J. Exper. Med., 1947, 85, 1.
19. Francis, T., Jr., Salk, J. E., and Brace, W. M., The protective effect of vaccination against epidemic influenza B. J. A. M. A., 1946, 131, 275.
20. Burnet, F. M., Cade, J. F. J., and Lush, D., Serological response to influenza virus infection during epidemic, with particular reference to sub-clinical infection. M. J. Australia, 1940, 1, 397.
21. Magill, T. P., and Sugg, J. Y., The significance of antigenic difference among strains of the A group of influenza viruses. J. Exper. Med., 1944, 80, 1.

THE VOLUME OF DISTRIBUTION OF MANNITOL AS A MEASURE OF THE VOLUME OF EXTRACELLULAR FLUID, WITH A STUDY OF THE MANNITOL METHOD[1]

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The clearance of mannitol has been advocated by Smith and his coworkers as a measure of glomerular filtration rate (1). Newman, Bordley and Winternitz (2) modified Smith's procedure by calculating the clearance of mannitol from the rate of fall of concentration in serum after a single injection of the substance and suggested that the rate of glomerular filtration is more logically related to the volume of extracellular fluid than to body surface area. They also presented evidence that the volume of distribution of mannitol is of approximately the same magnitude as this fluid compartment.

The thesis of Newman, Bordley and Winternitz that the clearance of mannitol can be calculated from the fall in serum concentration alone is valid only if mannitol reaches a constant volume of distribution in the body. The determination of the volume of distribution rests on the following assumptions: (*1*) that mannitol is neither formed nor destroyed in the body, (*2*) that it is distributed uniformly throughout some portion of the body water, (*3*) that its concentration in water of serum is a fair sample of its concentration throughout this portion, and (*4*) that it is excreted solely in the urine (3). It is the purpose of this investigation to determine whether the apparent volume of distribution of mannitol meets the above basic assumptions, and to study the relation of this volume to that of extracellular fluid.

Such a study requires a precise knowledge of the errors involved in the determination of mannitol. For this reason the method of determination and its accuracy were investigated in this laboratory and certain modifications introduced to minimize the error.

[1] Aided by a grant from the Fluid Research Fund of Yale University.

METHODS

The method of determination of mannitol used was that introduced by Smith, Finkelstein, and Smith (1), with modifications as indicated below. The method involves the oxidation of glucose-free filtrate with the reduction of a known excess of KIO_4, and quantitation of the remaining KIO_4 by iodometric titration with $Na_2S_2O_3$.

Reagents.

- Acid cadmium sulfate: 34.68 grams $3CdSO_4.8H_2O$ and 169.10 ml. 1 *N* H_2SO_4 made up to 1000 ml. H_2O.
- Sodium hydroxide 1.1 *N*.
- Baker's starch-free yeast: approximately 20 per cent suspension.
- Mannitol[2] standard solution: 100 mgm. per 100 ml.
- Acid potassium periodate: 0.60 gram KIO_4 and 20 ml. conc. H_2SO_4 made up to 1000 ml. H_2O.[3]
- Concentrated KI: approximately 50 per cent solution.
- Amylose: 1 per cent solution.
- Sodium thiosulfate: 0.005 *N* $Na_2S_2O_3$ made from 0.1 *N* stock solution.

Analysis in serum. The sample of serum is rendered glucose-free by fermentation with freshly washed starch-free baker's yeast in approximately 20 per cent suspension. To 2 ml. of serum in a 150 × 20 mm. pyrex tube are added 6 ml. of the yeast suspension which is thoroughly shaken before the withdrawal of each aliquot. The tubes are stoppered, shaken 3 times during 15 minutes and centrifuged. The exact extracellular fluid content of the yeast suspension is determined by centrifugation in a Wintrobe hematocrit tube. In 19 comparisons of the extracellular fluid content of the yeast suspension before and after yeasting a large number of serum and urine ali-

[2] The mannitol was supplied through the courtesy of Sharp and Dohme, Inc.

[3] Since this study was done Barker and Clark (15) have reported that free para-aminohippuric acid (PAH) reduces acid KIO_4 and so introduces an error in the determination of mannitol when present in the same biological media. These workers obviated the error by acetylating the PAH and acidifying the KIO_4 solution with acetic instead of sulfuric acid. While such an error does not affect the study reported here, it must be considered when clearances of mannitol and PAH are measured simultaneously.

quots, the mean difference was insignificant, being only 0.14 per cent with a maximum difference of 0.3 per cent. Filtrates of serum containing concentrations of mannitol of 50 mgm. per cent or less (including serum and reagent blanks) are fortified by the addition of a 2-ml. aliquot of 100 mgm. per cent aqueous solution of mannitol added to the 2 ml. of serum and 6 ml. of yeast. Filtrates of serum containing concentrations of 150 to 175 mgm. per cent and 175 to 200 mgm. per cent similarly are diluted with 2 ml. and 4 ml. of water, respectively. Failure to fortify or dilute resulted in increase of error: in 6 unfortified known solutions of mannitol in serum the average deviation was found to be +3.3 mgm. per cent, and in 3 undiluted solutions it was −5.0 mgm. per cent.

Four ml. of the supernatant yeasted serum are pipetted directly from the centrifuge tube into a similar tube containing 6 ml. of cadmium sulfate. To this are added 2 ml. of 1.1 *N* NaOH, the tubes are stoppered, shaken several times during 10 minutes, and centrifuged. The supernatant fluid is filtered through washed absorbent cotton (plugged in the neck of a glass funnel). Duplicate 2-ml. aliquots of the cadmium filtrate are pipetted into a pyrex tube (200 × 25 mm.) containing exactly 5 ml. of KIO_4 measured from an automatic pipette. Glass tears are placed in the tubes which are then heated in a boiling water bath for exactly 20 minutes. At the end of this time the tubes in the racks are transferred directly to a bath of ice water (5° C.) in which they are kept until titrated. Cooling in a water bath at room temperature (25° C.) definitely permits continued oxidation-reduction and results in false high values for mannitol if an hour or more elapses before titration: in 13 known solutions of serum cooled at room temperature the average deviation was +4.3 mgm. per cent.

The tubes and glass tears are washed down with water. Immediately before starting the titration about 1 ml. of approximately 50 per cent KI is added to each tube. This KI must be made up freshly each day. The partially reduced KIO_4, stirred with CO_2 bubbles, is titrated by means of a Bang burette equipped with a B-D needle tip for immersion titration, with 0.005 *N* $Na_2S_2O_3$ prepared freshly each day from a factored stock solution of 0.1 *N* $Na_2S_2O_3$. This burette must never be washed with dichromate cleaning solution. Traces of dichromate remaining after the routine 5 or 6 rinsings reacted with the $Na_2S_2O_3$ and were the largest single source of error between duplicate titrations. Approximately 0.5 ml. of 1 per cent amylose solution is added when the titration is within 0.25 ml. to 0.15 ml. of the endpoint. The endpoint can be read to 0.005 ml.

To calculate the concentration of mannitol it is first necessary to determine the amount present in the solution titrated. This value which is V in Equation 1 below, varies according to whether or not 2 ml. of standard solution of mannitol or 2 or 4 ml. of water are added to the yeasting solution and also according to the extracellular fluid content (h) of the 6-ml. aliquot of the particular yeast suspension used:

(*a*) $V = 1.33$ per $(2 + 6h)$
(*b*) $V = 1.33$ per $(4 + 6h)$
(*c*) $V = 1.33$ per $(6 + 6h)$

Each 1 ml. of 0.005 *N* $Na_2S_2O_3$ titrated by difference is equivalent to 0.092 mgm. of mannitol in the solution titrated. The concentration of mannitol (M) in mgm. per 100 ml. of the solution oxidized by KIO_4 is:

$$M = \frac{9.2f(A - B)}{V}, \qquad \text{Equation 1}$$

where: A = No. of ml. of $Na_2S_2O_3$ titrated against the blank KIO_4 solution.
B = No. of ml. of $Na_2S_2O_3$ titrated against the reduced KIO_4.
f = factor of the 0.005 *N* $Na_2S_2O_3$.
V = volume in ml. of the known solution added to the KIO_4.

Substituting V as calculated above, M is calculated as follows:

(*a*) $M = 6.9\ (2 + 6h)\ f\ (A - B)$, if no standard solution or water is added; 2
(*b*) $M = 6.9\ (4 + 6h)\ f\ (A - B)$, if 2 ml. of standard solution or water is added; 3
(*c*) $M = 6.9\ (6 + 6h)\ f\ (A - B)$, if 4 ml. of standard solution or water is added. 4

These equations (Equations 2, 3, 4) are the basic ones used to calculate the concentration of mannitol in serum or urine. Where 2 ml. of 100 mgm. per cent standard solution of mannitol has been added, 100 must be subtracted from the result. In all serum determinations the total serum blank must be subtracted from M as determined in these equations, to give the true value in serum. This blank is the value for non-fermentable reducing substance in the blank serum (net serum blank) plus the reagent blank. The reagent blank is the value for such substance in the yeast suspension and the cadmium sulfate and sodium hydroxide precipitating reagents. In all sera from fasting subjects the average for the total blank was 11.7 mgm. per cent with a range of 7.3 to 16.0 mgm. per cent, while the average net serum blank was 11.3 mgm. per cent with a range of 7.3 to 15.1 mgm. per cent. In 14 determinations the average reagent blank was +0.4 mgm. per cent with a range of −3.0 to +4.7 mgm. per cent.

In the analysis of 21 solutions of mannitol in serum of known concentrations between 25 and 200 mgm. per cent, the results of the observed concentrations were as follows: standard deviation = ±1.2 mgm. per cent, average deviation = 0.9 mgm. per cent, maximum deviation = 2.6 mgm. per cent. The mean difference between 54 pairs of duplicate titrations was 0.023 ml.

Analysis in urine. Urine is analyzed for mannitol in the same manner as serum, including the yeasting and precipitation. The only differences lie in the preliminary dilution of the urine and in the calculation of the blanks.

The high concentrations of mannitol in urine necessitate dilution before yeasting. By dividing the assumed glomerular filtration rate by the volume of urine in ml. per minute the approximate dilution factor is obtained which will reduce the concentration in urine to that simultaneously present in serum. If the latter value can be approximately estimated, the dilution factor can be adjusted to bring the concentration of mannitol in the diluted urine to lie between 50 and 150 mgm. per cent; further dilution or fortification is thereby avoided.

The net urine blank which must be subtracted to obtain an accurate estimation of the concentration of mannitol in urine, presents some difficulty when different urine samples are collected over intervals of time. The blank concentration of non-fermentable reducing substances varies from that in the initial specimen as the rate of urine flow varies and probably as the subject changes from a fasting to a post-prandial state. The most accurate value for the urine blank for each urine specimen (C_2), therefore would appear to be the blank concentration in the initial blank specimen (C_1) multiplied by a factor consisting of the rate of urine flow of the initial specimen (V_1) divided by that of the subsequent specimen (V_2):

$$C_2 = \frac{V_1C_1}{V_2}. \qquad \text{Equation 5}$$

In an experiment on a normal fasting subject receiving no mannitol in which variations in rate of urine flow were induced over a range of 0.83 to 7.00 ml. per minute, the observed blank concentration differed from the proportional concentration, as calculated by the above equation, by less than ½ the standard deviation of the method for urine. This method of calculating the net urine blank values between subjects can only be made in relation to rate of urine flow. In 8 blank urine specimens collected at various times under fasting conditions from the 3 normal subjects used in the present experiments, the average excretion rate of blank (non-fermentable reducing) substance was 1.9 mgm. per minute, with a range of 0.8 to 3.8 mgm. per minute.

To calculate the concentration of mannitol in urine, the reagent blank first must be subtracted from M as calculated by Equation 2. The value so obtained for the diluted urine is then multiplied by the dilution factor and the net urine blank subtracted. This gives the net concentration of mannitol in the original sample of urine.

The results of the determination of mannitol in 12 known solutions in urine ranging from 168 to 5077 mgm. per cent were as follows: standard deviation $= \pm 88$ mgm. per cent, average deviation $= 72$ mgm. per cent, maximum deviation $= 168$ mgm. per cent. When the deviation in each urine sample was divided by the dilution factor used, the average deviation was 2.7 mgm. per cent.

Summary. The modifications introduced into this method to improve its accuracy are listed as follows: (*1*) fortification of solutions containing low concentrations of mannitol, (*2*) cooling at 5° C. of the partially reduced KIO_4 solution between heating and titration, (*3*) avoidance of the use of dichromate cleaning solution in the titrating burette, (*4*) immersion titration using amylose rather than starch as an indicator, (*5*) calculation of the net urine blank from the rate of urine flow.

EXPERIMENTAL PROCEDURE

Three young male physicians in good health served as *normal subjects.* On arising in the morning the bladder was emptied and then ½ to 1 hour later, just before the injection of mannitol, the bladder was again emptied yielding the urine specimen for blank determination. These normal subjects were not catheterized. The subjects were fasting until the end of the fourth hour after injection, food and fluids were then taken *ad libitum.* During the preliminary period for obtaining the blank urine specimen, 2 cups of black coffee were allowed; thereafter till the fourth hour, 100 ml. of water per ½ hour were drunk immediately after each blood and urine collection.

The subjects were weighed stripped, 15 ml. of blood taken for the serum blank determination, and 12.0 to 25.9 grams of mannitol in a 25 per cent solution were injected intravenously from a calibrated syringe.[2] At the end of each period the bladder was emptied and 15 ml. of venous blood were taken from the arm opposite to that in which the mannitol was given. During the entire experiment the subjects were on their feet doing their usual work around the laboratory. With the exception of Experiments 1 and 2 which were 5 days apart, the intervals between experiments in each subject ranged from 3 to 9 weeks.

One other "normal" subject (M. W.) is included in Table I. This patient was a control to the above 3 subjects in that he was catheterized and his bladder was washed at the time of each urine collection. He was a 25 year old negro male receiving penicillin therapy for latent lues but with a normal state of hydration as far as could be ascertained.

Two patients with excessive accumulations of body fluids served as *abnormal subjects.* Distribution studies were done by the same technique outlined above before and after removal of part of the excess fluid. Both patients were catheterized and the bladder washed at each urine collection. The chloride balance was determined for the periods between the mannitol distribution studies. Patient L. M. (Experiments 9 and 10) was a 55 year old white male with massive ascites due to portal cirrhosis and carcinoma of the liver who was studied before and after a paracentesis. Patient F. J. (Experiments 11, 12, and 13) was a 60 year old white female with peripheral edema due to hypertensive heart disease. This patient on bed rest, digitalis, and a low salt diet, was given 6 grams of ammonium chloride daily and 3 intramuscular injections of *Mercuhydrin.* Mannitol distribution studies were done before and after the first mercurial injection and again at the end of a 10-day period during which she received 2 more injections.

METHODS OF CALCULATION

The apparent volume of distribution (V) of mannitol was calculated as follows:

$$V = \frac{A}{C},$$

where A equals the net amount retained in the body, and C equals the concentration of mannitol in serum water. The serum water content was determined by direct weighing of serum before and after drying. The mannitol space (E_M) is taken to be that volume of distribution of mannitol which remains constant within experimental error between two points of determination.

Chloride concentration in serum was determined by the method of Hald (4) and in urine by the Volhard-Harvey titration (5). The change in chloride space (ΔE_{Cl}) was calculated from the chloride balance (b_{Cl}) and the extracellular water chloride concentrations (Cl_1 and Cl_2) as described elsewhere (6), using the initial mannitol space (E_{M_1}) as E_1:

$$E_{Cl_2} = \frac{(E_1 Cl_1) + b_{Cl}}{Cl_2},$$

$$\Delta E_{Cl} = E_{Cl_2} - E_1.$$

RESULTS IN NORMAL SUBJECTS

Recovery of mannitol. In 3 experiments in 2 subjects the mannitol recovered in the urine 8 hours after injection was 89, 93, and 99 per cent of that injected; 24 to 26 hours after injection the recovery was 154,101, and 108 per cent (Table I). Mannitol evidently was not destroyed. The greater than 100 per cent recovery at 24 to 26 hours is probably due to increased excretion of non-fermentable reducing substances after the fasting period (*i.e.*, an unmeasurable increase in urine blank). Unpredictable changes in the urine blank in this period do not affect the calculation of volume of distribution of mannitol. It is unlikely that this increased blank is mannitol formed in the body, but direct proof of this is lacking.

Attainment of equilibrium. When the concentrations of mannitol in serum water are plotted semi-logarithmically against time after injection, the concentration at ½ to ¾ hour lies above the straight line passing through the subsequent points, in 6 of the 8 experiments. That is, the rate of all in concentration was greater between ½ hour and 1 hour than in the later periods. This is strong evidence that equilibrium in mannitol concentration was attained through the "mannitol space" between ½ and 1 hour in these experiments. In Experiment 1, equilibrium appeared to have been reached by ½ hour, and in Experiment 3 not until some time between 1 and 1½ hours. These variations may be related to the fact that it was in these experiments that the smallest and largest doses of mannitol, respectively, were given.

Volume of distribution. In 5 of the 8 experiments the volume of distribution of mannitol remained essentially unchanged between 1 and 3 hours after injection (Table I); after 3 hours the analytical error was too great to draw any conclusion. In 2 of the other experiments it appeared to expand. In Experiment 1, however, the small dose of mannitol rendered the error of the determination after 1 hour too large to be definitive. In Experiment 5 there was either a true but slight increase in the volume of distribution or the subject failed to empty his bladder completely. In Experiment 8 the volume of distribution was not determined after 1¼ hours. In all of the experiments the distribution volume at ½ hour was significantly lower than at 1 hour, due to the fact that equilibrium had not been attained (see above).

Agreement between the volumes of distribution at 1 hour after successive injections was remarkably good in each subject, the widest variations being not more than 0.06 per cent of the body weight or 4 per cent of the absolute volume. Uniformity between volumes of distribution of the different subjects at 1 hour was not so great; the range was 18.7 to 23.0 per cent of the body weight.

The error of the determination of the volume of distribution is complicated in that it is the resultant of 2 different analytical errors: those of serum and urine. If these 2 errors are in opposite directions they tend to cancel, if in the same direction they augment each other. Although there is 1 chance in 2 that the latter occurred in each instance, the degree of error in each volume of distribution has been calculated which would result from an error of the standard deviation in the same direction in serum and urine. The value thus obtained is designated as the *maximum standard deviation error* of the volume of distribution.

The magnitude of the error so calculated at any one time is the direct resultant of the concentration of mannitol in serum and the concentration in, and volume of, urine. The lower the concentrations are or the larger the urine volume is, the greater is the error. Thus, the error is

TABLE I

The volume of distribution and clearance of mannitol in normal subjects

Experiment	Subject	Weight and surface area	Time after injection of mannitol	Mannitol injected	Urine		Mannitol net retained	Serum		Serum H_2O	Volume of distribution			Glomerular filtration	
					Volume	Mannitol content		Mannitol conc.	H_2O content	Mannitol conc.	Vol.	Max. σ error*	Vol.	Rate	Max. σ error*
		kgm. sq. m.	*hours*	*grams*	*ml.*	*mgm.*	*mgm.*	*mgm. per cent*	*grams per liter*	*mgm. per liter*	*liters*		*per cent body weight*	*ml. per min. per 1.73 sq. m.*	
1	R. E.	70.17	0	12.95											
		1.84	½		108	3,145	9,805	76.2	932	817	12.0	±0.3	17.1		
			1		84	1,923	7,876	55.9		600	13.1	±0.4	18.7	87	± 4
			2		285	2,475	5,397	30.4		326	16.5	±1.5	23.5	92	±11
			3		174	1,785	3,609	19.1	932	205	17.6	±2.1	25.1	120	±11
			24		1,344	10,565	−6,959	0.3		3					
2	R. E.	69.85	0	20.75											
		1.83	½		102	4,780	15,970	122.3	935	1,307	12.2	±0.2	17.5		
			1		60	2,935	13,025	90.0		963	13.5	±0.3	19.3	88	± 2
			1½		58	2,292	10,726	73.4		787	13.6	±0.3	19.5	85	± 4
			2		43	1,563	9,157	64.3	933	689	13.3	±0.3	19.0	75	± 4
			3		81	2,440	6,712	39.2		420	16.0	±0.7	22.9	76	± 3
			8		252	4,440	2,267	12.1		130	17.4	±3.8	24.9	61	± 3
3	R. E.	70.78	0	25.85											
		1.84	½		124	5,565	20,285	156.6	936	1,673	12.1	±0.2	17.1		
			1		89	3,885	16,397	112.9		1,207	13.6	±0.2	19.2	90	± 2
			1½		100	2,768	13,610	88.2		943	14.4	±0.3	20.3	87	± 4
			2		209	2,172	11,431	73.0		781	14.6	±0.5	20.6	84	± 9
			3		250	3,210	8,215	52.4	933	562	14.6	±0.8	20.6	81	± 8
			8		547	6,470	1,738	11.2		120	14.5	±5.0	20.5	76	± 7
			26		928	1,923	−186	2.7							
4	J. R.	62.13	0	20.75											
		1.73	½		91	4,770	15,980	128.2	937	1,367	11.7	±0.2	18.8		
			1		85	4,260	11,710	80.7		862	13.6	±0.3	21.9	119	± 4
			1½		59	2,410	9,294	63.5		679	13.7	±0.3	22.1	112	± 5
			2		62	2,005	7,284	49.7		532	13.7	±0.4	22.1	120	± 6
			3		188	2,810	4,470	30.6	932	329	13.6	±1.1	21.9	118	± 6
			8		282	4,290	175								
			25		830	1,792	−1,618								
5	J. R.	62.48	0	25.85											
		1.73	½		175	6,190	19,660	143.3		1,534	12.8	±0.2	20.5		
			1		121	4,220	15,429	102.7	934	1,100	14.0	±0.3	22.4	115	± 4
			1½		92	2,765	12,656	80.2		858	14.7	±0.4	23.5	101	± 5
			2		69	2,100	10,550	61.9		663	15.9	±0.4	25.5	97	± 5
6	L. G.	67.63	0	20.75											
		1.78	½		86	4,900	15,850	106.6	944	1,138	13.9	±0.2	20.5		
			1		49	2,855	12,986	79.0		841	15.4	±0.3	22.8	103	± 2
			1½		50	2,480	10,500	64.4		687	15.3	±0.4	22.6	109	± 4
			2		42	1,802	8,793	53.9		577	15.3	±0.4	22.6	99	± 6
			3		67	2,500	6,289	38.3	934	410	15.3	±0.7	22.6	90	± 5
7	L. G.	68.46	0	25.55											
		1.79	½		141	6,310	19,240	126.5	932	1,357	14.2	±0.3	20.8		
			1		84	3,890	15,340	90.9		975	15.7	±0.3	23.0	121	± 3
			1½		102	2,892	12,441	72.3		778	16.0	±0.4	23.4	114	± 6
			2		112	2,320	10,115	58.8		632	16.0	±0.5	23.4	115	± 8
			3		282	3,075	7,035	39.0	930	419	16.8	±1.2	24.6	102	±12
8	M. W.	62.20	0	25.75											
			¾		365	8,920	16,819	132.6	932	1,423	11.8	±0.4	19.0		
			1		89	1,570	15,241	101.9		1,093	14.0	±0.3	22.5	89	± 5
			1¼		128	1,680	13,554	88.4		949	14.3	±0.3	23.0	116	± 8

* Maximum standard deviation error (see text).

greater with a smaller dose of mannitol (Experiment 1) than with a larger dose. Given approximately the same dosage, the lower the amounts of mannitol retained, the lower are the concentrations in serum and urine, and hence the greater is the error. In Experiments 2 to 8, inclusive, in normal subjects the maximum standard deviation error of the volume of distribution does not exceed ± 0.5 liter until only 44 per cent or less is retained; after this period the error greatly increases in magnitude. Finally, given the same dosage and the same proportion of mannitol retained, the larger the urine volume the greater the error (Experiments 8 to 12).

For the volumes of distribution calculated at 1 hour after injection of 20.7 to 25.9 grams of mannitol, the average maximum standard deviation error was ± 0.3 liter or ± 2.1 per cent of the volume calculated. In these experiments the deviation from uniformity of the calculated volumes of distribution between 1 and 8 hours after injection could be explained by this maximum standard deviation error, with 2 exceptions only (Experiment 2, 3 hours, and Experiment 5, 2 hours).

Glomerular filtration rate. The mannitol clearance or glomerular filtration rate in the 3 subjects ranged from 75 to 121 ml. per minute per 1.73 square meters of body surface (Table I). These values are considerably below those given by Goldring and Chasis (7) for normal males, namely, 131 ± 22 ml. per minute per 1.73 square meters, but our subjects were not at rest. The maximum standard deviation error for the 1 to 1½ hour periods averaged ± 5 ml. per minute or ± 4.8 per cent of the clearance, and increased as the excretion rate diminished.

RESULTS IN ABNORMAL SUBJECTS

Attainment of equilibrium. In the 2 subjects with abnormal accumulation of body fluid, as might be predicted, equilibrium throughout the mannitol space was not reached as soon as in the normal subjects (Table II). In the patient with ascites, equilibrium apparently was not reached at 4 hours after injection in Experiment 9, before paracentesis; or at 3 hours in Experiment 10, after paracentesis. In Experiment 9 the concentration of mannitol in ascitic fluid water was determined directly at 4½ hours and found to be 50.4 mgm. per cent; the concentration in serum water at 4 hours was 52.3 mgm. per cent. These findings suggest that equilibrium actually had been reached at 4 hours but was not confirmed by a subsequent point. In the patient with cardiac edema, equilibrium appeared to be attained at 4, 3 and 2 hours after injection as the edema progressively diminished.

Volume of distribution. In both of these subjects before any removal of excess fluid, the mannitol space or volume of distribution, after approximate equilibrium had been reached, was a much larger proportion of the total body weight than it was in the normal subjects, namely 37.6 per cent of the body weight in L. M. with ascites and 40.7 per cent in F. J. with cardiac edema (Table II).

In both patients the change in mannitol space (ΔE_M) was compared with the change calculated for the chloride space (ΔE_{Cl}). In patient L. M. the volume of cell-free ascitic fluid removed by paracentesis was 4.9 liters, ΔE_{Cl} was − 4.9 liters, and ΔE_M was − 3.0 maximum σ error ± 0.7 liter. In patient F. J. the check between ΔE_M and ΔE_{Cl} was very good for Experiments 11 and 12 which were done before and immediately after the first mercurial diuresis. ΔE_{Cl} was − 5.6 liters and ΔE_M was − 6.0 maximum σ error ± 0.8 liter. The agreement between the 2 in Experiments 12 and 13, done 10 days apart, was not so close, ΔE_{Cl} being − 5.3 liters and ΔE_M being − 6.9 liters. The agreement of ΔE_M with the change in weight, − 6.6 kgm., was better, and suggested that a cumulative positive error existed in the chloride balance, probably due to failure of complete absorption of the 6 grams of enteric coated NH_4Cl ingested daily. Such an error, of course, would be considerably less in the 24-hour period between Experiments 11 and 12. It should be noted that between Experiments 11 and 13 in which period $\Delta E_M = -12.9$ liters, the patient's 4 + pitting edema of the legs, sacrum and lower abdominal wall almost completely disappeared.

Glomerular filtration rate. The range of the mannitol clearance, 35 to 69 ml. per minute per 1.73 square meter, was definitely lower in these 2 patients than in the normal subjects. No change in clearance occurred in patient L. M. be-

TABLE 11

The volume of distribution and clearance of mannitol in subjects with abnormal accumulations of body fluids

Experiment	Subject and weight	Date	Time after injection of mannitol	Mannitol injected	Urine		Ascitic fluid		Mannitol net retained	Chloride balance	Serum			Glomerular filtration		Mannitol volume of distribution		Change in:	
					Volume	Mannitol content	Volume	Mannitol conc.			Mannitol conc.	Chloride conc.	H_2O content	Rate	Max. error*	Vol.	Max. error*	Mannitol space	Chloride space
	kgm.		hours	grams	ml.	mgm.	ml.	mgm. per cent	mgm.	m. eq.	mgm. per cent	m. eq. per liter	grams per liter	ml. per min. per 1.73 sq. m.		liters		liters	
Patient with ascites due to cirrhosis and carcinoma of liver: before and after paracentesis																			
9	L. M. 61.2	Dec. 5	0	25.45								94.6							
			1		137	5,965			19,485		101.6		928			17.8	±0.3		
			2		68	3,120			16,357		76.4			63	± 1	19.9	±0.4		
			3		65	2,590	30	43.9	13,748		59.9		928	63	± 1	21.3	±0.5		
			4		56	1,730			12,013		48.5			65	± 2	23.0	±0.7		
			4½				4,900†	49.1											
10	56.4	Dec. 6	0	22.85	415					−518		90.0							−4.9
			1		160	6,780			16,070		86.7		927			17.2	±0.4		
			2	"	67	2,295			13,767		68.0			69	± 2	18.8	±0.4		
			3		80	2,480			11,283		52.2			62	± 2	20.0	±0.6	−3.0	
Patient with peripheral edema due to hypertensive heart disease: before and after mercurial diuresis																			
11	F. J. 72.6	Dec. 17	0									101.7	936						
			1	25.45	183	3,110			22,340		96.7					21.6	±0.1		
			2		108	1,900			20,432		73.9			38	± 3	25.9	±0.3		
			3		90	1,485			18,941		61.8			35	± 3	28.6	±0.4		
			4		102	1,635			17,301		54.9			47	± 4	29.5	±0.5		
12	65.3	Dec. 18	0		7,250					−772		97.5	938						−5.6
			1	25.45	700	5,730			19,720		98.8					18.7	±1.0		
			2		340	2,785			16,927		73.6			61	±10	21.6	±0.7		
			3		172	1,342			15,579		60.8			35	± 5	24.0	±0.7		
			4		186	1,552			14,022		56.1			46	± 5	23.5	±0.8	−6.0	
13	58.7	Dec. 28	0							−419		103.5	931						−5.3
			1	25.45	187	5,710			19,740		118.9					15.5	±0.3		
			2		106	3,170			16,560		95.4			55	± 2	16.2	±0.3		
			3		89	2,680			13,872		77.6			54	± 3	16.6	±0.4	−6.9	
			4		76	1,970			11,896		66.3			54	± 3	16.7	±0.4		

* Maximum standard deviation error (see text).

† Water content equaled 974 grams per liter.

fore and after paracentesis. However, in patient F. J. the clearance was higher in Experiment 13 when most of her edema had been removed.

DISCUSSION

The evidence presented in these experiments indicates that in the normal subject mannitol is distributed throughout a definite fraction of the body fluids. This fraction remains essentially constant between 1 and 3 hours after intravenous injection and is reproducible in the same subject after repeated injections. The assumptions necessary to the calculation of a constant volume of distribution, as outlined in the beginning of this paper, are supported by the data presented. Mannitol was completely recovered in urine. It appeared to be uniformly distributed throughout 1 portion of the body fluids, and the concentrations of mannitol in ascitic fluid checked very closely with that in serum, when equilibrium probably had been attained.

The time required in normal subjects for equilibrium to be reached between serum and the rest of the mannitol space was found in these experiments to lie between ½ and 1 hour. This is definitely longer than the 20 minutes reported by Newman, Bordley, and Winternitz (2), but approximates the time of 1 hour reported by Alving and Miller (8) for inulin. Certainly for any measurement of the mannitol space it is essential that this equilibrium be reached, as indicated by a constant value for the volume of distribution between 2 separate points. In patients with abnormal accumulations of extracellular fluid, the time for equilibration may be greatly extended. In patient L. M. with ascites, it was only approached at 3 to 4 hours. In patient F. J. with edema, equilibrium was reached more rapidly as

the edema and mannitol space diminished in size, namely, at 3 to 4 hours, 2 to 3 hours, and 1 to 2 hours (Experiments 11, 12, and 13). Prolongation of the time for equilibration probably occurs in the presence of any type of circulatory impairment.

No direct evidence is presented regarding the relation between the absolute mannitol space and the extracellular fluid. In the normal subjects the magnitude of the former approximated the accepted value for the volume of the latter, namely, ⅕ of the body weight. The range in these experiments was 18.7 to 23.0 per cent of the body weight. These values are slightly higher than the average for 11 normal subjects found by Newman, Bordley and Winternitz (2) which was 17.7 per cent of the body weight. The volume of distribution of sucrose, a similar polysaccharide, was shown by Lavietes, Bourdillon and Klinghoffer (9) to range in 3 normal subjects between 17.2 and 20.3 per cent of the body weight.

No one substance has ever been shown to be completely excluded from all body cells. Due to the relatively small amounts of chloride in tissues (10), radioactive chloride, Cl^{38}, is probably the best reference substance yet found. In dogs the volume of distribution of Cl^{38} has been demonstrated to be smaller than those of radioactive sodium, Na^{24}, and sulfocyanate (11). However, the very short life of Cl^{38} makes it an extremely difficult substance to use experimentally. The author knows of only 1 human subject in whom the volume of distribution of Cl^{38} has been determined: the value obtained by Moore (12) was 18.9 per cent of the body weight. As the volume of distribution of Na^{24} in the same subject was 26.5 per cent of the body weight, it is obvious that radioactive sodium is no more useful as a reference substance in man than in the dog. Kaltreider, Meneely, Allen and Bale (13) obtained an average value of 24.8 per cent of body weight in 14 normal subjects for the volume of distribution of Na^{24}, 3 hours after injection. This value is close to that of Moore's stated above. In the absence of a simultaneous study of the volumes of distribution of Cl^{38} and of mannitol in the same subjects, it can only be stated that the range of mannitol volumes of distribution found in our experiments lies between those of Cl^{38} and Na^{24} as found by these other workers in other subjects. This is hardly definitive.

Studies of the phase distribution of sugars in individual tissues have not been extensive. Mannitol does not penetrate erythrocytes (1, 2). Wilde (14) demonstrated that inulin and sucrose were confined to a phase in skeletal muscle smaller than that of chloride. These tissue studies appear to confirm the results of the volume of distribution studies as indicating that mannitol and allied polysaccharides are in the main confined to extracellular fluid.

Another type of evidence is available in our experiments on the patients with ascites and edema. The *change* in mannitol space approximated the change in chloride space as calculated from the balances of and changes in serum concentration of chloride. This evidence does not indicate that the absolute size of the mannitol space is the same as that of chloride (11), but it does suggest that determination of the mannitol space may be a useful and independent measure of *changes* in extracellular fluid volume. Furthermore calculation of such change from the changes in balance and serum concentrations of chloride is possible only if the initial extracellular fluid volume, E_1, is known. In a normal subject this value can be estimated reasonably from the body weight. But in patients with dehydration or edema some direct measurement of E_1 is necessary. Direct determination of the mannitol space may be used for this purpose.

Successive determinations of the volume of distribution of mannitol in the same subject require some consideration of the rate of excretion of this substance. The rapidity of excretion probably prevents the detection of significant changes in the extracellular fluid volume between multiple points determined, following a single injection. On the other hand, the substance must be completely excreted before volumes of distribution can be determined following a second injection. Residual amounts in the serum and urine would introduce an unknown error into the calculation of blank concentrations, and hence into the calculation of the distribution volume.

The errors have also been studied which are involved in the measurement of the mannitol clearance, or the rate of glomerular filtration.

Theoretically, when the mannitol clearance is calculated for the ½- to 1-hour period, when the serum concentration of mannitol is higher than that in the interstitial fluid, it does not measure the glomerular filtration rate alone. Part of the removal of mannitol from the plasma is extrarenal and hence the clearance value should be higher than the true renal clearance. The data in Table I, however, reveal that the clearances in this period were not significantly higher than the succeeding period (with the possible exception of Experiment 5). Failure to reach complete equilibrium, therefore, did not introduce a significant error into the determination of the rate of glomerular filtration.

Newman, Bordley, and Winternitz (2) propose to calculate the clearance of mannitol from the rate of fall in serum concentration only, thus obviating the necessity of accurate urine collections and analyses. To do so, however, the volume of the extracellular fluid or mannitol space must be known. No other method of measuring extracellular fluid volume appears to be readily available, and its measurement by determination of the volume of distribution of mannitol requires the analysis of urine for mannitol.

These workers also state the opinion that the mannitol clearance or glomerular filtration rate is more significant physiologically if it is quantitated per unit of extracellular fluid volume than per unit of body surface or merely per unit of time. This seems to the author to be erroneous in that the size of the reservoir containing a substance to be excreted has no relation to rate at which the kidney is able to excrete it. The kidney can only act on that portion of the extracellular fluid volume which runs through it, the plasma. Thus in comparing the mannitol clearance between 2 and 3 hours after injections in Experiments 11 and 13, in terms of ml. per minute it rose from 35 to 49 (+ 40 per cent); in terms of ml. per minute per 1.73 square meters of surface area it rose from 35 to 54 (+ 55 per cent); and in terms of ml. per minute per liter of extracellular fluid volume (mannitol space) it rose from 1.2 to 3.0 (+ 150 per cent). In each experiment it was the same pair of kidneys; *i.e.*, per unit of kidney the change in filtration rate was only + 40 per cent. Because the extracellular fluid volume happened to be reduced by almost ½, it does not seem significant from the standpoint of renal function to correct the change in rate to + 150 per cent.

SUMMARY

In normal human subjects following a single intravenous injection of mannitol:

1. Mannitol was fully recovered in the urine in 24 hours.
2. Equilibrium appeared to be complete between ½ and 1 hour after injection.
3. The volume of distribution was constant between 1 and 3 hours after injection, ranged between 18.7 and 23.0 per cent of the body weight, and was reproducible in each subject following repeated injections.
4. Under specified conditions the maximum standard deviation error in the volume of distribution did not exceed ± 0.5 liter.

In 2 patients with ascites and edema following a single injection of mannitol:

1. Four hours or more were required for diffusion to equilibrium.
2. Equilibration was speeded as edema was reduced.
3. The decrease in mannitol space compared favorably with the decrease in chloride space as produced by paracentesis and mercurial diuresis.

The glomerular filtration rates in both groups of subjects were determined. The error due to failure of attainment of complete equilibrium was less than that due to the analytical error.

The relation of the mannitol space to the extracellular fluid is discussed.

Certain modifications in the mannitol method are described.

BIBLIOGRAPHY

1. Smith, W. W., Finkelstein, N., and Smith, H. W., Renal excretion of hexitols (sorbitol, mannitol, and dulcitol) and their derivatives (sorbitan, isomannide, and sorbide) and of endogenous creatinine-like chromogen in dog and man. J. Biol. Chem., 1940, 135, 231.
2. Newman, E. V., Bordley, J., III, and Winternitz, J., The interrelationships of glomerular filtration rate (mannitol clearance), extracellular fluid volume, surface area of the body, and plasma concentration of mannitol: a definition of extracellular

fluid clearance determined by following plasma concentration after a single injection of mannitol. Bull. Johns Hopkins Hosp., 1944, 75, 253.

3. Winkler, A. W., and Smith, P. K., The apparent volume of distribution of potassium injected intravenously. J. Biol. Chem., 1938, 124, 589.
4. Hald, P. M. in Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Williams and Wilkins, Baltimore, 1932, Vol. II, Methods, p. 838.
5. Harvey, S. C., The quantitative determination of the chlorides in the urine. Arch. Int. Med., 1910, 6, 12.
6. Elkinton, J. R., and Winkler, A. W., Transfers of intracellular potassium in experimental dehydration. J. Clin. Invest., 1944, 23, 93.
7. Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease. The Commonwealth Fund, New York, 1944.
8. Alving, H. S., and Miller, B. F., A practical method for the measurement of the glomerular filtration rate (inulin clearance). Arch. Int. Med., 1940, 66, 306.
9. Lavietes, P. H., Bourdillon, J., and Klinghoffer, K. A., The volume of extracellular fluids of the body. J. Clin. Invest., 1936, 15, 261.
10. Manery, J. F., and Hastings, A. B., The distribution of electrolytes in mammalian tissues. J. Biol. Chem., 1939, 127, 657.
11. Winkler, A. W., Elkinton, J. R., and Eisenman, A. J., Comparison of sulfocyanate with radioactive chloride and sodium in the measurement of extracellular fluid. Am. J. Physiol., 1943, 139, 239.
12. Moore, F. D., Determination of total body water and solids with isotopes. Science, 1946, 104, 157.
13. Kaltreider, N. L., Meneely, G. R., Allen, J. R., and Bale, W. F., Determination of the volume of the extracellulor fluid of the body with radioactive sodium. J. Exp. Med., 1941, 74, 569.
14. Wilde, W. S., The chloride equilibrium in muscle. Am. J. Physiol., 1945, 143, 666.
15. Barker, H. G., and Clark, J. K., Effect of para-aminohippurate on mannitol determinations by periodate-iodide-thiosulfate method. Proc. Soc. Exp. Biol. & Med., 1947, 64, 120.

EFFECTS OF EXPERIMENTAL HYPERTONIA ON CIRCULATING LEUKOCYTES [1]

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During the 50 years that have elapsed since Arneth originally studied the behavior of circulating leukocytes, many substances and physical factors have been shown to exert an effect of either depressing or stimulating the total numbers of circulating white cells. Most of the substances have been protein in nature. Thus bacterial products (1), peptone (2), enzymes (3), tissue extracts (4), and pseudoglobulins (5), have all been shown to cause temporary alteration in the peripheral leukocyte picture. Among the physical factors that can similarly influence white cell circulation are cold (6) and ultraviolet light (7). Boyd (8), in 1913, was the first to show that inorganic ions can stimulate an increase in the total white count. While treating insanity with intravenous salt solution, he noted an increase in the polymorphonuclear cells of the blood. Later Bluemel and Lewis (9), in 1924, were able to demonstrate a transitory leukocytosis under the same circumstances. Beard and Beard (10), in 1928, working with rabbits, showed that the intravenous injection of salt caused a transitory leukopenia followed by a leukocytosis. They postulated that the cation Na^+ might play some role in regulation of white counts.

While working with leukocyte tissue cultures, it was observed (11) that polymorphonuclear neutrophiles exhibited a lengthened survival in hypertonic media whereas lymphocytes showed a corresponding slightly lengthened survival in hypotonic media. Although such studies dealt solely with cell survival, they raised interesting possibilities of correlation with *in vivo* effects on regulation of circulating leukocytes. The present study was therefore undertaken to determine whether simple alteration in the tonicity of serum would similarly effect leukocyte maturation and release into the peripheral circulation. That is: would experimental hypertonicity induce a selective polymorphonuclear leukocytosis?

METHODS

Subjects: Healthy 10- to 14-kilogram dogs. Hypertonicity was induced, both acutely and chronically, by diverse means. Prior to the start of any 1 experiment, the animals were maintained on a standard diet and fluid intake for 1 week. In experiments which extended over a several-day period, all blood studies were done at the same time of day, under the same circumstances and by the same set of observers, to minimize, as far as possible, the effects of activity, excitement and variation in the diurnal tide on the peripheral leukocyte pattern. Total white counts and smears were made with standard techniques. Red cell counts and/or hematocrits were done throughout each experiment. All variations in absolute leukocyte counts were thus corrected for the factor of hemoconcentration. All variations in tonicity of the extracellular fluid were determined by the technique of freezing point depression on defibrinated blood and are reported in milliosmoles per liter (conversion factor: $\Delta T_f = 1.86$ M).

CHRONIC HYPERTONICITY

Chronic hypertonicity was attained over a 5- to 10-day period by the following means: (*1*) Chronic water and food deprivation; (*2*) chronic water deprivation in the presence of a dry protein diet; (*3*) chronic water deprivation in the presence of a dry protein diet plus hypertonic salt solution parenterally. As shown by Danowski (12), and others, the diuretic effect of the end products of nitrogen metabolism hastened the onset of chronic hypertonia. Chronic hypertonia was effected 1 time on each of 3 seperate dogs, "A," "C" and "F."

RESULTS

The average resting (pre-experimental) molarity was 308 milliosmoles. The levels of hypertonicity that were attained varied from 364 to 484 [3] milliosmoles. In each instance the hyper-

[1] The author is indebted to Miss Jean Sunderlin for technical assistance.

[2] Donner Foundation, Research Fellow.

[3] When this exceptionally high molarity was reached the dog (Exp. A-2) was in extremis and convulsing. Immediately after the tests were made, the dog was successfully revived by large amounts of hypotonic fluid i.v.

tonicity was accompanied by an increase in the absolute numbers of circulating neutrophiles. The lymphocytes and eosinophiles remained essentially unchanged. The absolute numbers of monocytes decreased. In Figures 1 and 2, the increase in absolute neutrophile counts are plotted against the rise in molarity which occurred in each of the 3 experiments, A-2, C-1, F-1. It will be seen that the molarity of the serum and the absolute neutrophile count bore a linear relation to one another. Under the experimental conditions which existed, the absolute numbers of circulating polymorphonuclear neutrophiles appeared to be a factor of the tonicity of the extracellular fluids.

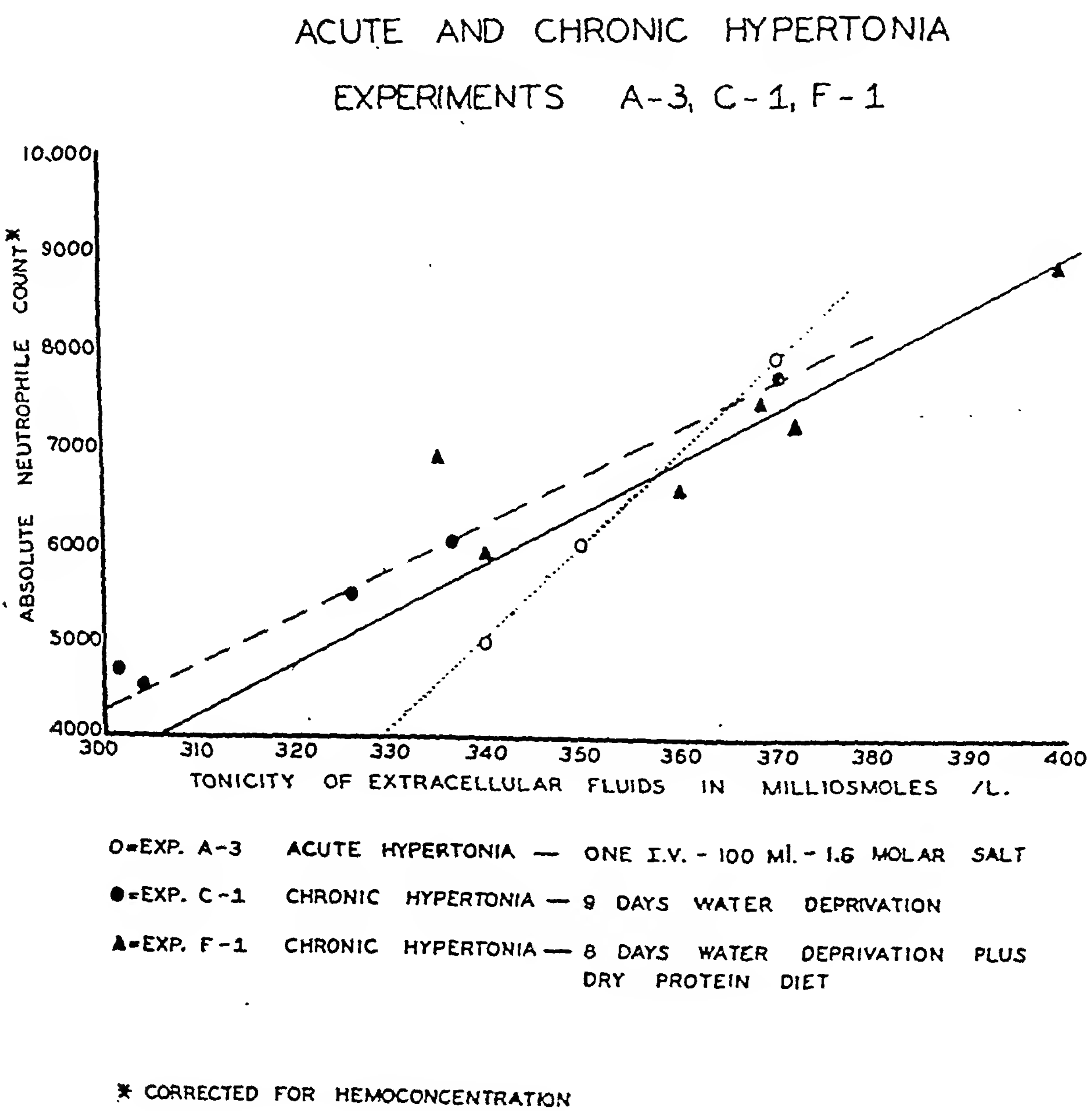

FIG. 1.

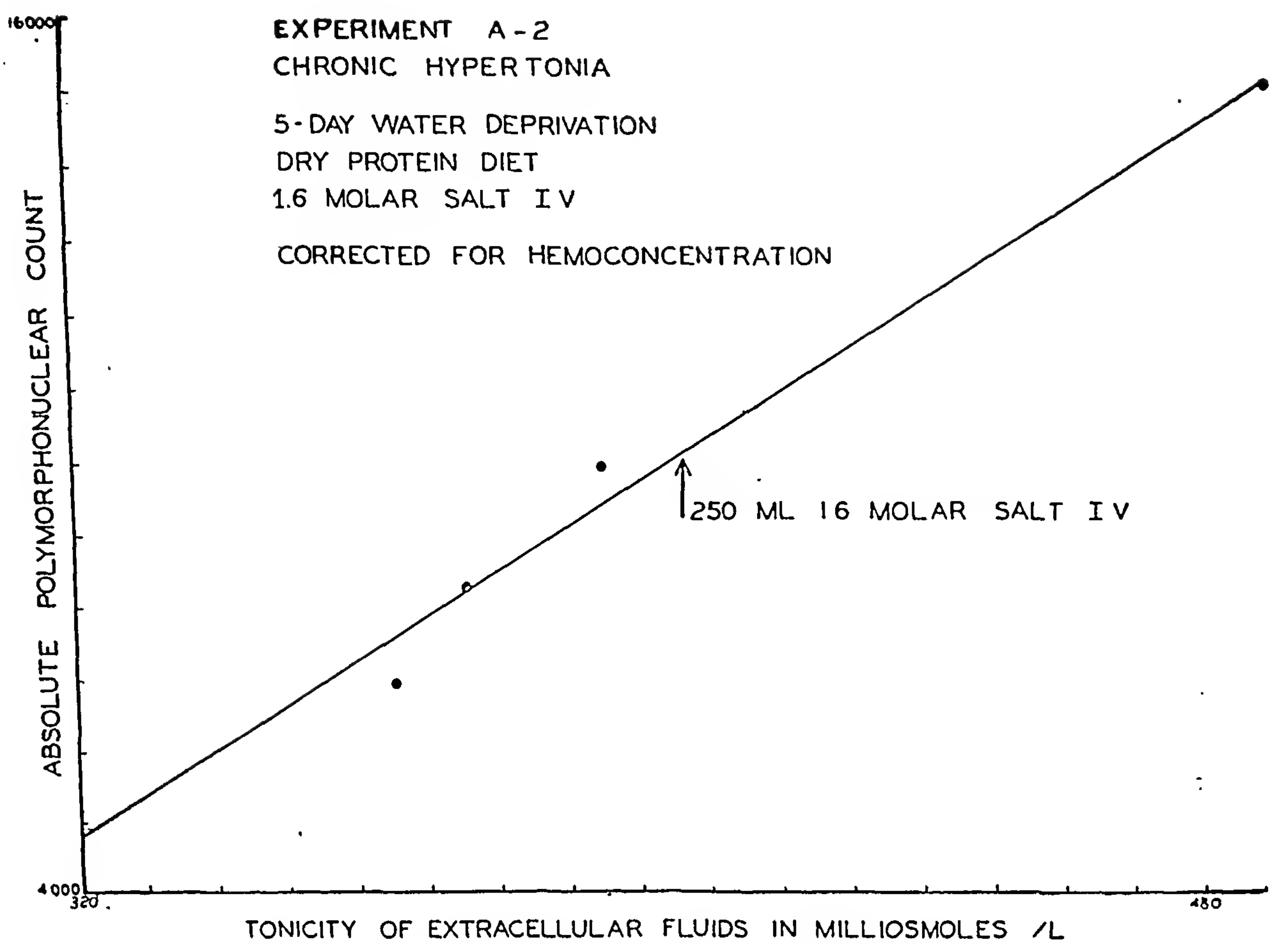

Fig. 2.

ACUTE HYPERTONICITY

Acute hypertonicity was produced, in all instances, by the intravenous administration of hypertonic sodium chloride solution. The experiment was performed 6 times on 2 animals, dogs "A" and "G." In Experiment A-3, 100 ml. of 1.6 molar sodium chloride were infused. In all other experiments, G-2, 3, 4, 6 and 7, 250 ml. of 1.0 molar sodium chloride solution were infused. If sufficient time was allowed for the administration of the infusions (45 to 90 minutes), this amount of salt was exceptionally well tolerated and produced no objective signs of physiologic disturbance. If, however, the rate of infusion was reduced to ¼ hour or less, profound cerebral irritability, clonic convulsions and death occurred.

RESULTS

Administration of hypertonic salt solution intravenously resulted in acute hypertonia. The molarity of the serum rose rapidly to an average level of 370 milliosmoles and returned to normal in about 7 hours. In all instances, the rise in molarity was followed by a selective increase in the absolute numbers of circulating neutrophiles.' The neutrophile rise was statistically significant and of an order of magnitude of 50 per cent or greater. Immediately following the infusion of the hypertonic salt solution, there was a slight decrease in the total numbers of all cell types, both red and white. This was felt to be due to a simple dilution effect from the volume of infused solution and not to be indicative of any specific action on blood cells. Then the rise in absolute numbers of granulocytes began abruptly, around ¼ to ½ hour after the infusion was completed, and reached its highest level in from 7 to 10 hours. In acute hypertonicity, as in chronic, the increase in absolute neutrophile count bore a linear relationship to the increase in molarity of the extracellular fluids (Exp. A-3, Figure 1). Since, with acute hypertonicity, the rise in molarity usually preceded by an hour or more the rise

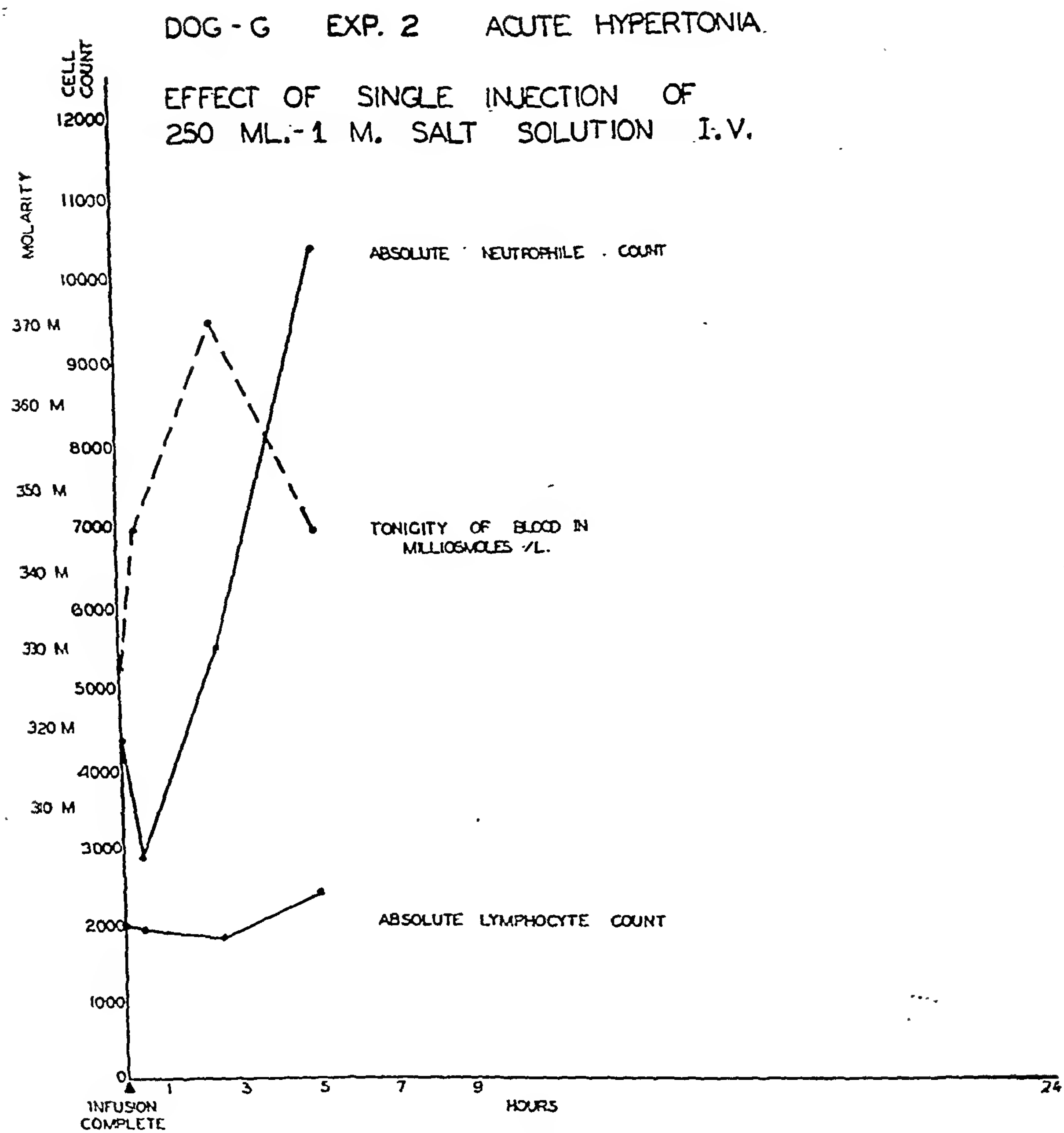

FIG. 3.

in neutrophiles, the fluctuations in count and tonicity are best plotted against time. Thus in Figures 3, 4 and 5, the 24-hour variation in absolute counts following a single infusion of 250 ml. of hypertonic saline may be seen for Experiments G-2, 3 and 4. As a control experiment it was next decided to test the effect of an infusion of a similar amount of isotonic saline to determine how much of the polymorphonuclear rise, if any, could be attributed to a non-specific "washing out" of cells secondary to an increased rate of blood flow.

In Experiment G-5 (Figure 6), it will be seen that a transitory rise in *neutrophile count did* occur after isotonic saline. The rise was slight, however, and not sustained. It was felt that part of this rise might be due to splenic contraction from the stimulus of the intravenous infusion. The animal was therefore splenectomized and the experiments repeated, with both isotonic and hypertonic saline.

In Experiment G-8 (Figure 7), it will be seen that removal of the spleen completely abolished the non-specific rise that occurred in the control injection of isotonic saline. However, in Experiments G-6 and 7 (Figures 8 and 9), it will be seen that removal of the spleen did not affect the rise in count which follows hypertonicity. The rise in neutrophile count, as previously, was

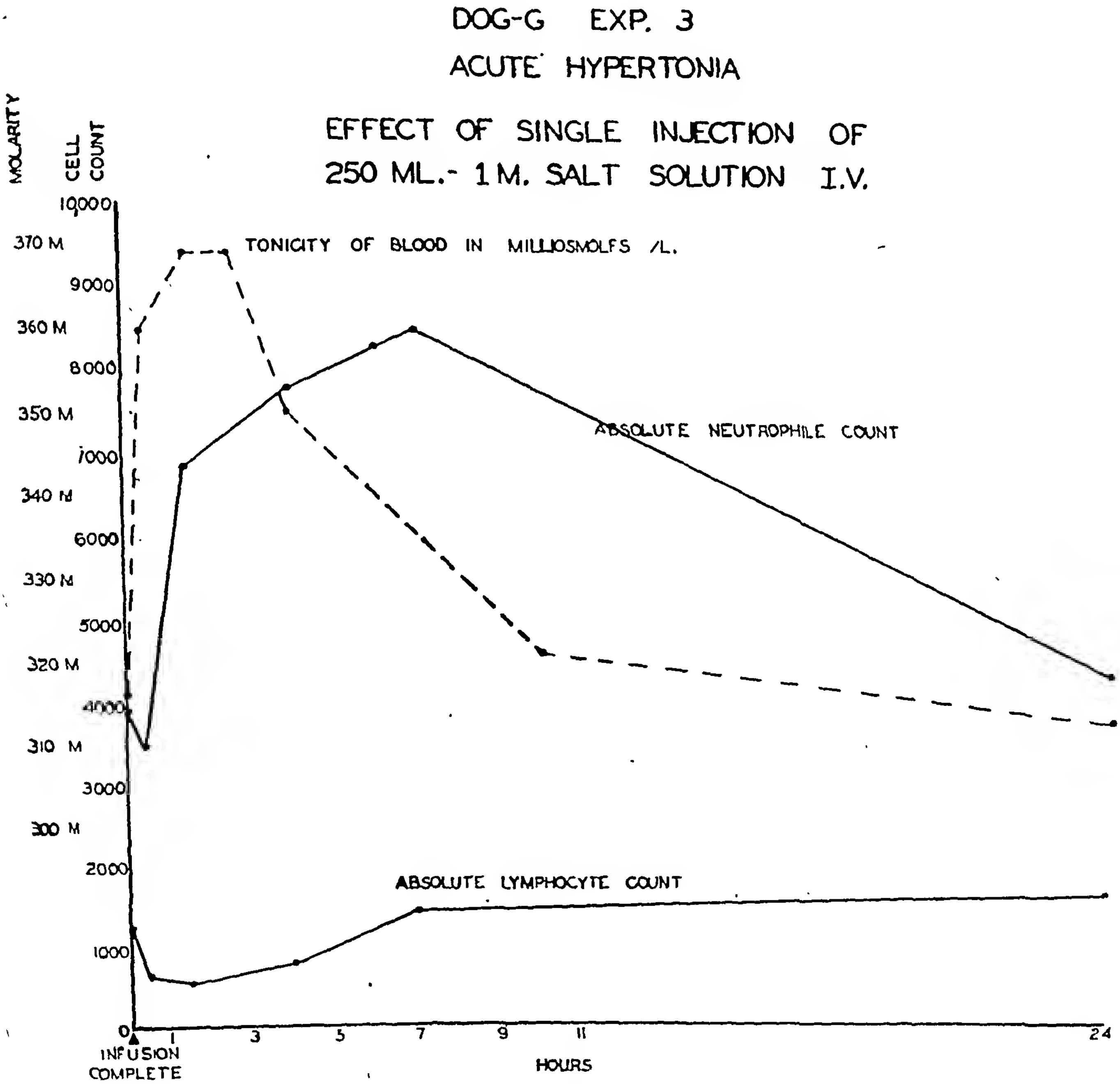

FIG. 4.

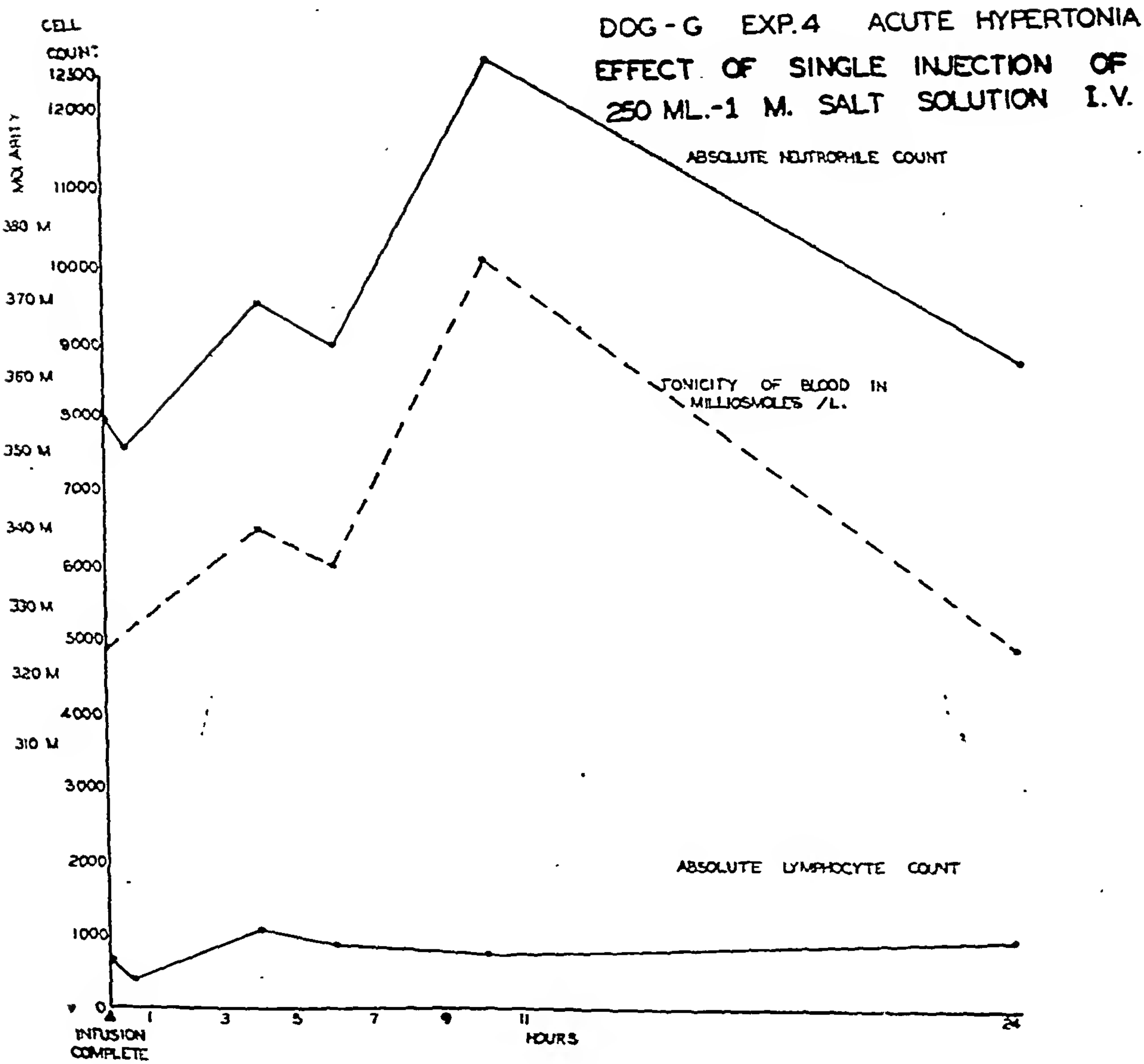

Fig. 5.

prompt, selective (unaccompanied by lymphocytosis) and statistically significant.

DISCUSSION

Within the limits of these experiments it is reasonable to ascribe a cause and effect relationship between the milliosmolar strength of the serum and the absolute numbers of circulating neutrophiles. Due to the fact that the leukocyte variations in these experiments were produced by alterations in osmotic pressure rather than through the use of foreign proteins or other substances alien to the blood stream, it is not unreasonable to postulate that variations in milliosmolar strength of the extracellular fluids may play a role in determining the leukocyte pattern in some diverse disease states. For example: (*1*) The unexplained polymorphonuclear leukocytosis which regularly accompanies the hypertonicity of diabetic coma. (*2*) The unexplained granulopenia and

relative lymphocytosis which regularly accompanies the hypotonicity of Addison's disease. Studies are currently being carried on to investigate these relationships.

The mode of action of the hypertonic polymorphonuclear leukocytosis produced in these experiments is unknown. Its prompt occurrence in acute hypertonicity makes it obvious that it is not due to the effect which originally stimulated these investigations, namely: The lengthened survival time of neutrophiles in hypertonic media (11). The recent work of Lawrence (13), who places the *in vivo* survival time of leukocytes at 16 hours, would similarly tend to make such an effect improbable. The 2 most likely mechanisms would seem to be either a release of preformed cells from some theoretical "store house" in the body or an accelerated maturation and release of immature cells from the bone marrow. If the first mechanism obtains, it would be difficult to visualize the absence of a simultaneous rise in all leukocyte types (lymphocytes, monocytes,

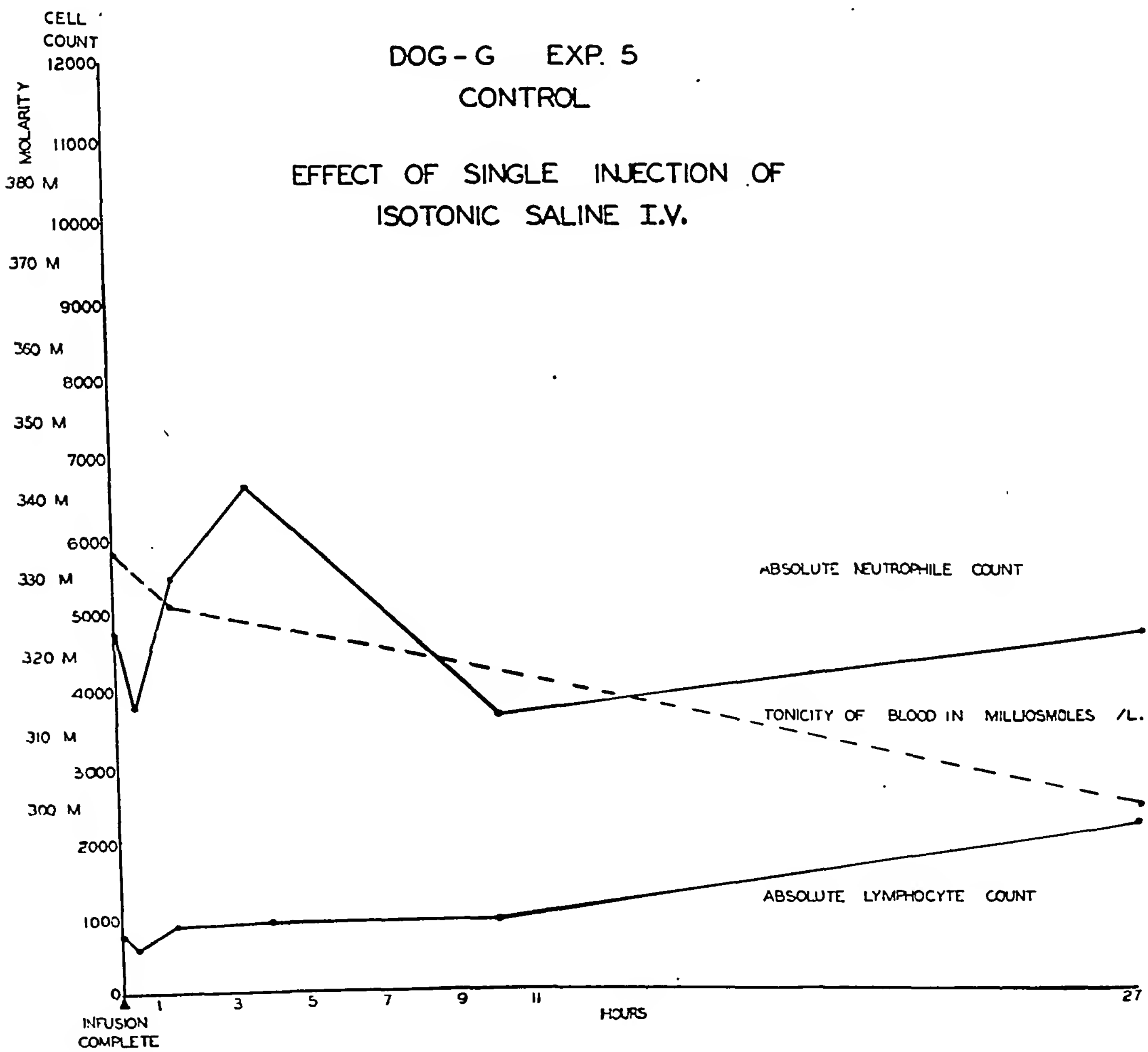

FIG. 6.

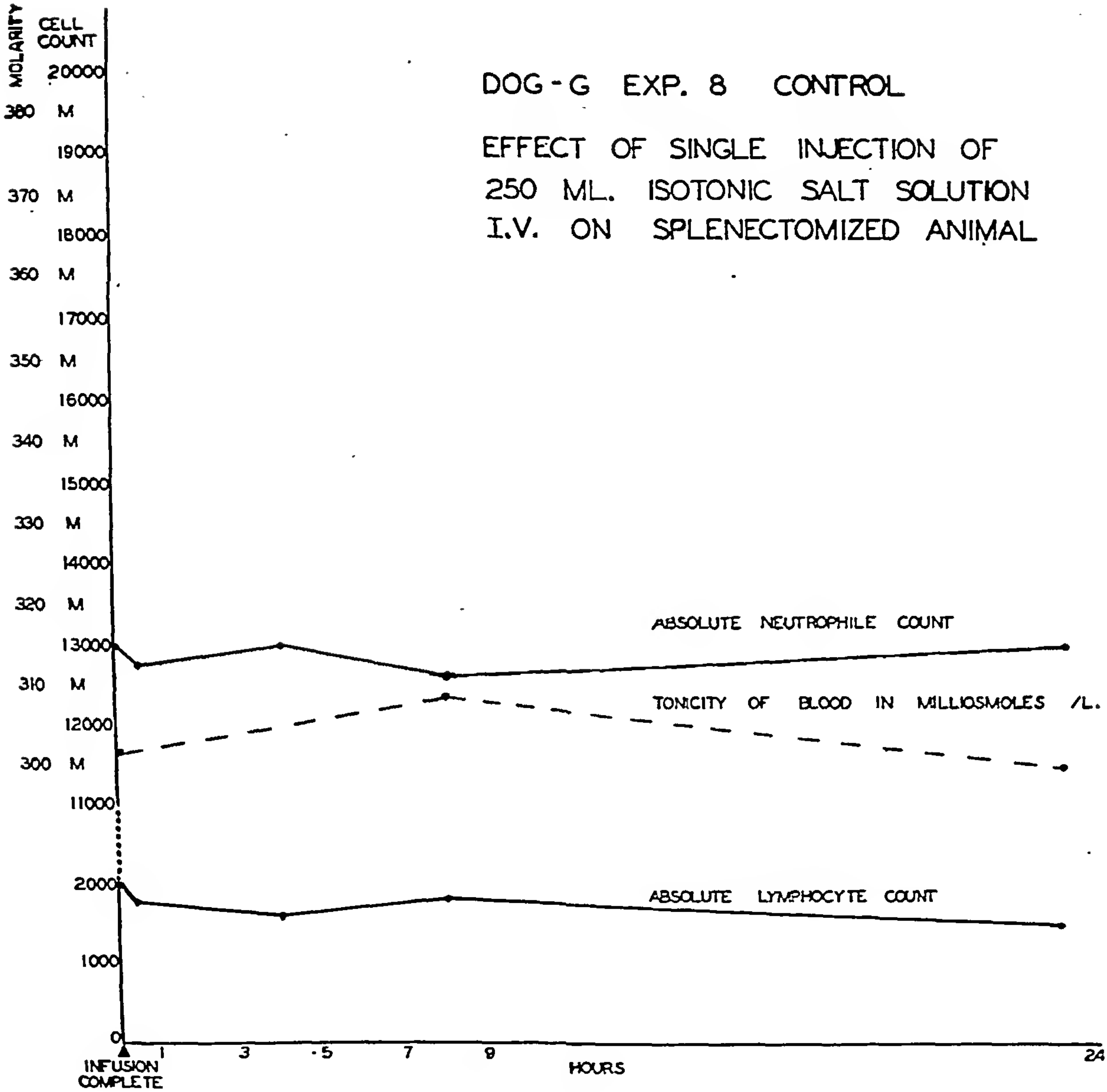

FIG. 7.

eosinophiles) and the persistence of the rise in the case of the chronically induced hypertonicity. If the second mechanism obtains, one would expect rather large numbers of juvenile neutrophiles to appear in the peripheral circulation. Although this phenomenon was looked for, and sometimes found, its appearance was too irregular to lend weight to this possibility. On the basis of these experiments, no explanation of the mechanism can be offered. Further investigation into the mode of action of hypertonic polymorphonuclear leukocytosis should include serial bone marrow observations.

SUMMARY

Hypertonicity of the serum of dogs was produced both acutely and chronically. In all in-

stances it was accompanied by relative and absolute increases in the numbers of circulating neutrophiles. The absolute numbers of lymphocytes remained essentially unchanged with increases in molarity. Monocytes, when present in numbers to be statistically significant, showed a slight decrease with increases in molarity.

CONCLUSION

Under the conditions of these experiments the increases in absolute numbers of circulating neutrophiles were directly proportional to the increases in the milliosmolar strength of the extracellular fluids.

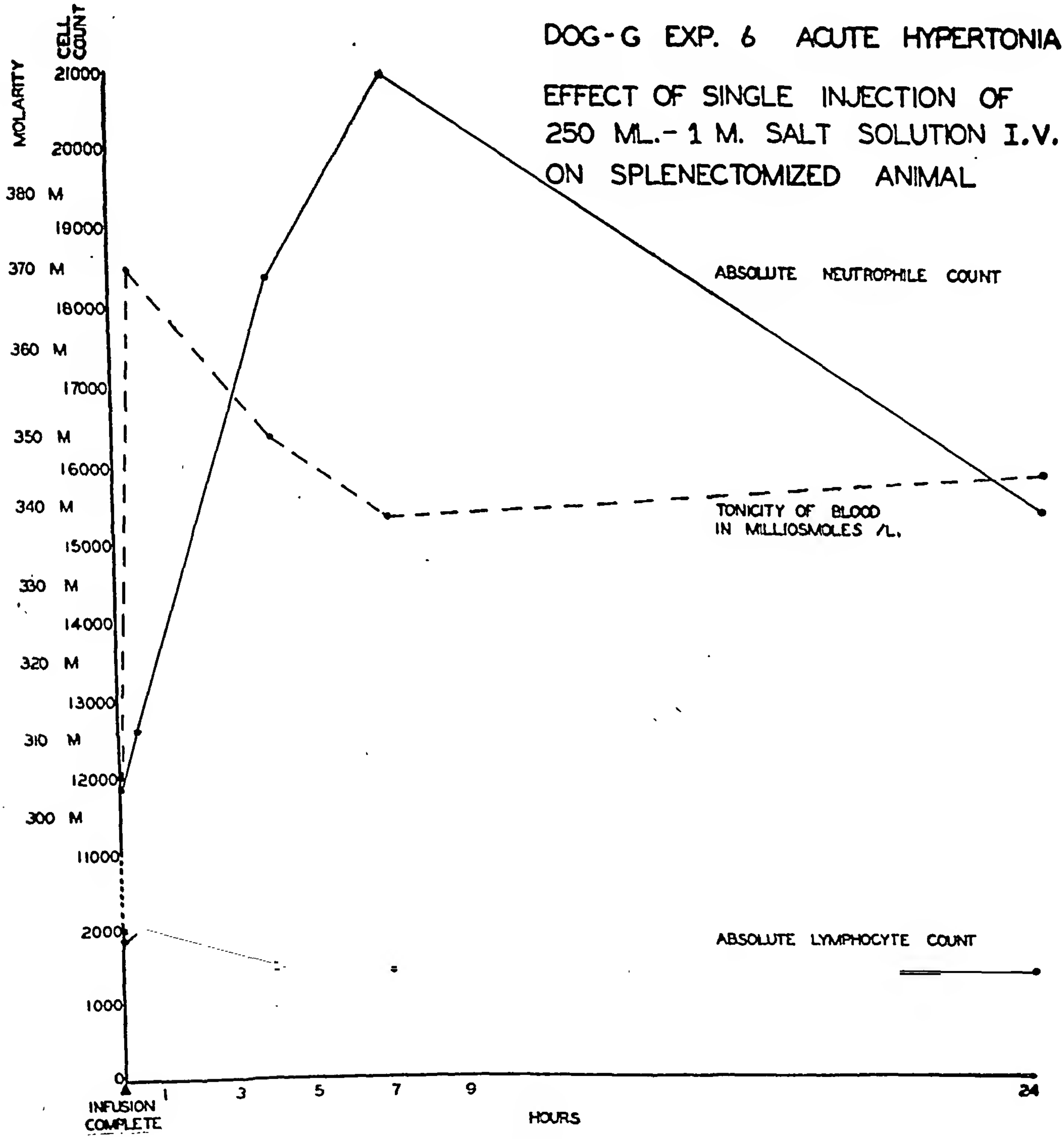

Fig. 8.

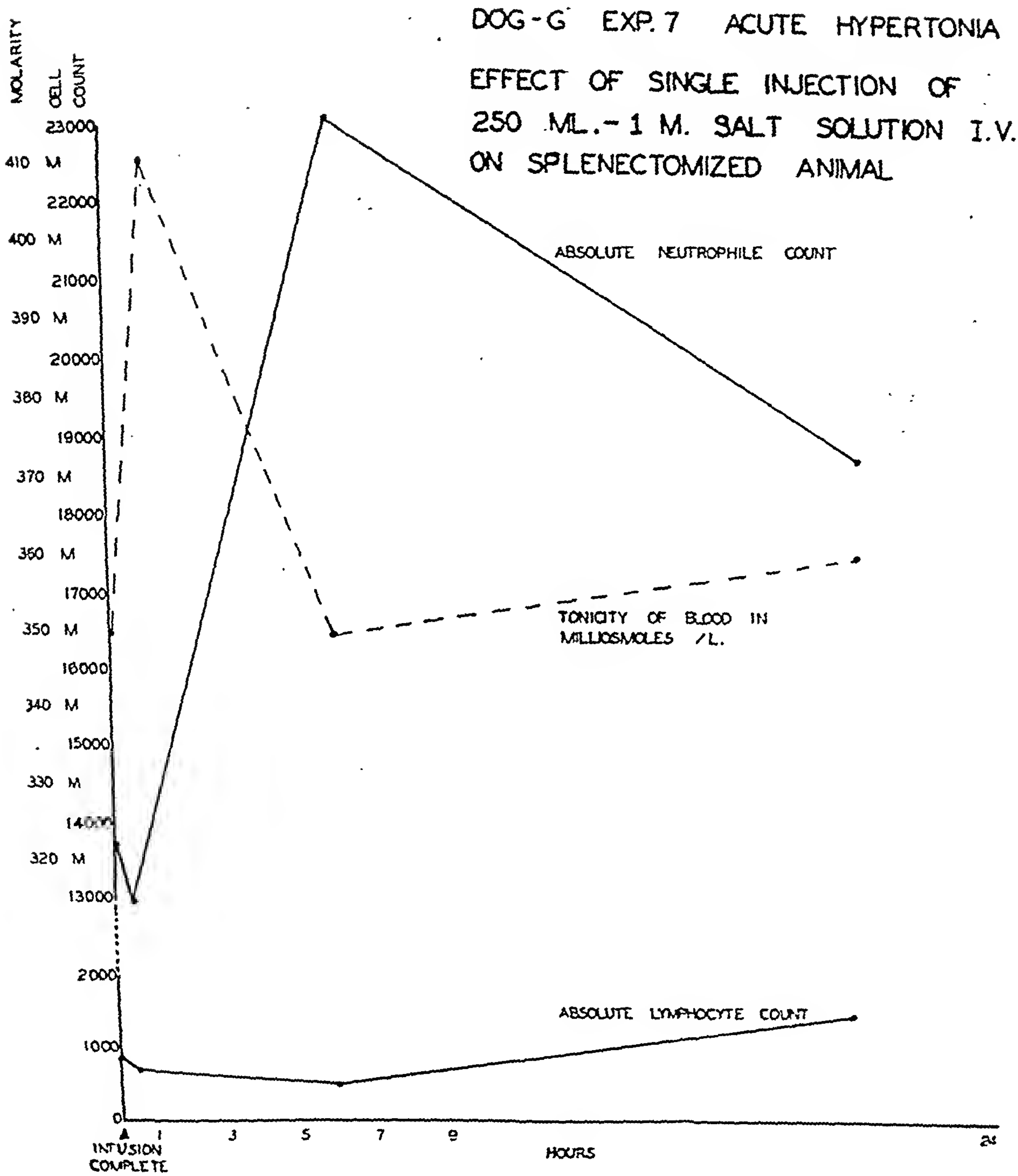
DOG-G EXP. 7 ACUTE HYPERTONIA
EFFECT OF SINGLE INJECTION OF 250 ML.-1 M. SALT SOLUTION I.V. ON SPLENECTOMIZED ANIMAL
MOLARITY
CELL COUNT
23000
410 M
22000
400 M
21000
390 M
20000
380 M
19000
370 M
18000
360 M
17000
350 M
16000
340 M
15000
330 M
14000
320 M
13000
2000
1000
0
ABSOLUTE NEUTROPHILE COUNT
TONICITY OF BLOOD IN MILLIOSMOLES /L.
ABSOLUTE LYMPHOCYTE COUNT
INFUSION COMPLETE
1
3
5
7
9
HOURS
24

FIG. 9.

BIBLIOGRAPHY

1. Ewing, J., Toxic hypoleukocytosis. N. Y. Med. J., 1895, **61**, 257.
2. Arneth, J., Die Qualitative Blutlehre, Druck Der Univ Ersitätsdruckerei H-Stürtz. A. G. Würzburg, Leipzig, 1920, 309–321.
3. Ponder, E., and Flint, K. N., Arneth count; effect of various drugs and extracts. Quart. J. Exper. Physiol., 1927, **16**, 393.
4. Nettleship, A., Leukocytosis associated with acute inflammation. Am. J. Clin. Path., 1938, **8**, 398.
5. Menkin, V., Dynamics of Inflammation. Macmillan, New York, 1940, 110–114.
6. Burnham, F. W. E., Hemocytes and Hemic Infections. New Werner Co., Akron, O., 1913, p. 58.
7. Kennedy, W. P., and Thompson, W. A. R., Studies on Arneth count; deflection of count by ultra-violet rays. Quart. J. Exper. Physiol., 1928, **18**, 263.
8. Boyd, W., Leucocytosis produced by the injection of normal saline solution. J. Ment. Sci., 1913, **59**, 86.
9. Bluemel, C. S., and Lewis, R., Effect of intravenous saline solution on the leukocyte count. Am. J. Physiol., 1924, **67**, 464.
10. Beard, L. A., and Beard, J. W., Effect of intravenous injection of sodium chloride on distribution of white blood cells in peripheral circulation. Am. J. Physiol., 1928, **85**, 169.
11. Tullis, J. L., Studies on permeability of the leucocyte. Am. J. Physiol., 1947, **148**, 708.
12. Danowski, T. S., Elkinton, J. R., and Winkler, A. W., Deleterious effect in dogs of dry protein ration. J. Clin. Invest., 1944, **23**, 816.
13. Lawrence, J. S., Ervin, D. M., and Wetrich, R. M., Life cycle of white blood cells; rate of disappearance of leukocytes from peripheral blood of leukopenic cats. Am. J. Physiol., 1945, **144**, 284.

THE RELATIONSHIP OF SODIUM CHLORIDE TO HYPERTENSION [1]

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Restriction of the sodium chloride intake as a therapeutic measure in hypertensive vascular disease was first advocated many years ago (1, 2, 3). Although subsequent investigators (4, 5, 6, 7) claimed that the addition or removal of salt from the diet failed to influence the blood pressure level significantly, interest in the subject has recently been renewed.

Selye and his coworkers (8) noted a striking hypertensive effect when sodium chloride was administered to experimental animals receiving injections of desoxycorticosterone acetate. Grollman and his associates (9) observed that drastic reduction in sodium intake resulted in a decline in blood pressure in some hypertensive patients. They believed it probable that the beneficial effects of the diet proposed by Kempner (10) might be due to the restriction of salt. Recently evidence has been presented (11) that sodium chloride potentiates the pressor activity of desoxycorticosterone acetate when injected into rats rendered nephritic with nephrotoxic serum.

The present study was undertaken to reexamine the relationship of sodium chloride to hypertensive vascular disease in man.

METHODS

Men and women with uncomplicated hypertensive vascular disease were studied on the wards of the Presbyterian Hospital and the Research Service, First Division, of the Goldwater Memorial Hospital. Subjects were included only if the antecedent blood pressure consistently exceeded 140/90 mm. of mercury and in the absence of cardiac pain or insufficiency, renal or cerebral complications or fever. All patients were free of albuminuria, showed normal phenolsulfonephthalein excretion and urine concentration tests, and in all instances the venous pressure was within normal limits.

Blood pressures were measured each morning in the same arm by the same observer, with the subject quiet and relaxed in bed, the head of which was raised to a 30° angle. At least 5 and usually 7 or 8 readings were taken at half-minute intervals and the lowest systolic and diastolic value recorded. This value was designated the *"resting"* blood pressure as compared to *"casual"* readings taken under various conditions of activity at other times during the day. In order to secure an adequate baseline, it was found obligatory to carry out preliminary observations for at least 3 weeks.

All subjects were kept in bed until after blood studies and blood pressure measurements were made, and ambulatory activity was standardized at a constant level the rest of each day. The patients were weighed daily before breakfast on the same scales. The daily fluid intake and urine output were recorded, distilled water being supplied for drinking purposes.

Throughout the study the patients were given a constant diet and fluid intake. Identical salt-poor daily menus were prepared. Repeated direct analyses of aliquots taken from an entire day's cooked diet gave values of between 0.25 to 0.35 grams of sodium or considerably less than 1 gram of sodium chloride per day. These salt-poor diets yielded 1,700 to 2,200 calories and 70 to 80 grams of protein, and did not include special ingredients such as dialyzed milk. Sodium chloride was administered by mouth using weighed salt shakers, additional supplements being given in some instances in the form of enteric-coated tablets. When indicated, desoxycorticosterone acetate [2] (DCA) was injected subcutaneously in doses of 5 mgm. twice daily.

The urine chloride was determined daily from the 24-hour collection, daily aliquots being measured at approximately weekly intervals for their sodium content. Fasting blood samples were obtained at intervals for CO_2 content, chloride, sodium, potassium, protein and urea nitrogen determinations. The serum volume was measured with the blue dye T. 1824. Weekly electrocardiograms were taken and in no instance showed evidence of significant myocardial damage or serial changes.

A sample chart (Figure 1) illustrates the general form

[1] This study was made possible through the generosity of the Albert and Mary Lasker Foundation. We are indebted to Miss Ann D. Barrows, dietitian, Miss Margaret G. Hawthorne, head nurse, Miss Katherine Vislocky, technician, John T. Sharp and Dominic G. Iezzoni for their invaluable assistance.

[2] Desoxycorticosterone acetate (Doca) was supplied through the courtesy of Dr. L. A. Pirk of Roche-Organon, Inc., Nutley, New Jersey.

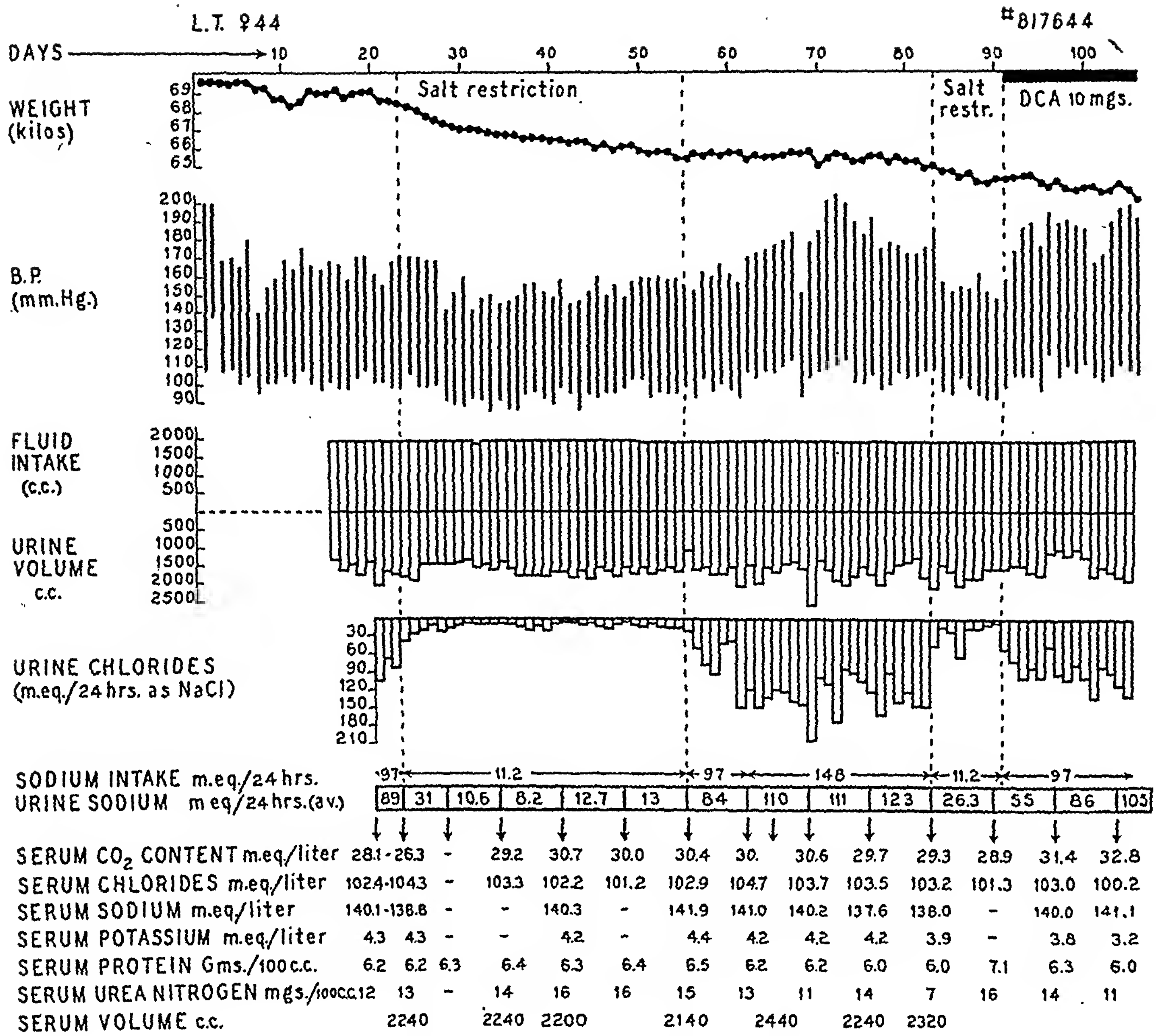


FIG. 1. SAMPLE CHART ILLUSTRATING GENERAL FORM OF INVESTIGATIONS ON EACH HYPERTENSIVE SUBJECT

in which subsequent investigations were conducted and recorded on each patient.

RESULTS

I. *Effect of rigid sodium chloride restriction on blood pressure.* Six hypertensive patients were studied following a preliminary baseline period and constant regimen which included 4 grams of sodium chloride added to the salt-poor diet. A decrease in *"resting"* blood pressure was observed in all patients in from 3 to 5 days after the withdrawal of the sodium chloride from the diet, with a return to previous levels only after sodium chloride administration was resumed (Figure 2, I). The majority of blood pressure readings, during the period of observation, remained above 140/90 mm. of mercury. *"Casual"* readings were apparently uninfluenced by sodium chloride restriction, and each patient exhibited the same variation, the same response to such stimuli as activity, apprehension or the cold pressor test as was demonstrated during the fore-period.

The withdrawal of sodium chloride from the diet for a period of 2 weeks was tolerated in these subjects without discomfort. Slight weight loss and small increases in urine output were generally observed. The decreased intake was sharply

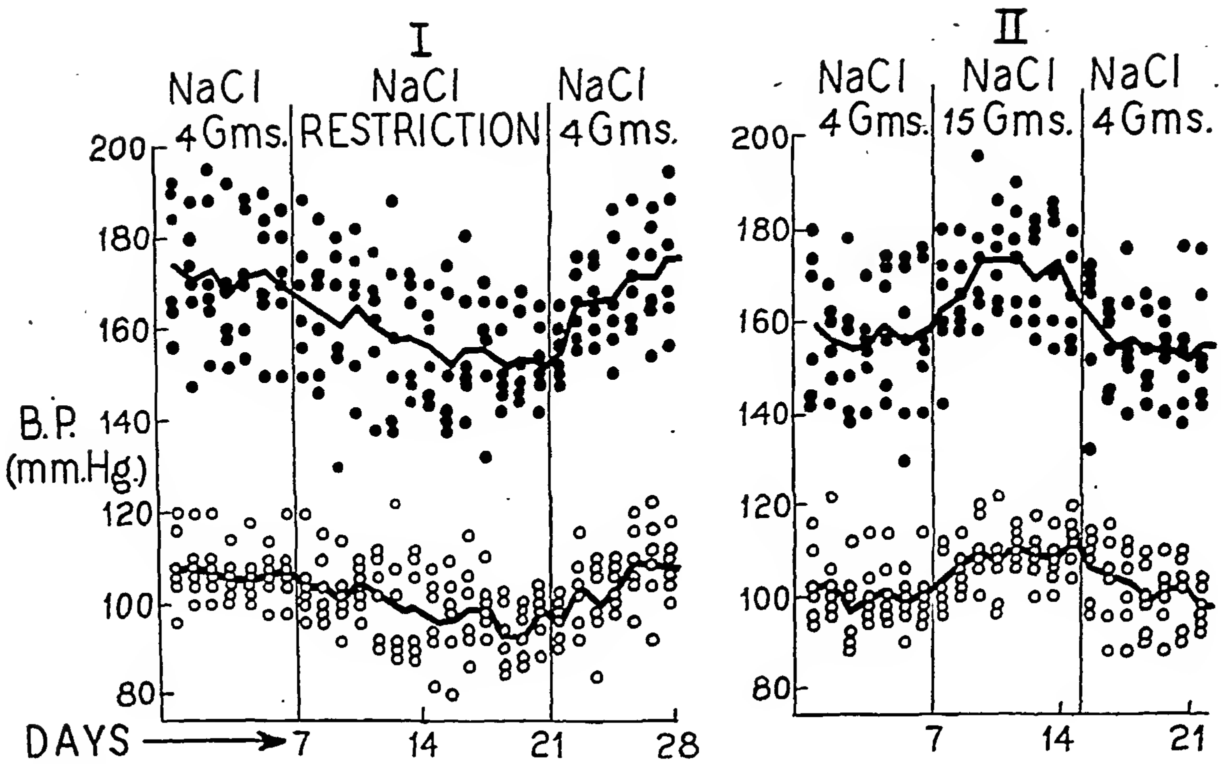


FIG. 2. EFFECT OF RIGID SODIUM CHLORIDE RESTRICTION AND OF INCREASED SODIUM CHLORIDE INTAKE ON 6 PATIENTS WITH HYPERTENSION

reflected in urine sodium and chloride levels, but serum CO_2, chloride, sodium, potassium, protein, urea nitrogen and volume measurements were not materially influenced (Table I). Ballistocardiographic tracings were unchanged on repeated records taken before and after sodium chloride restriction in 2 of these patients, both of whom exhibited a significant drop in blood pressure on salt restriction.

II. *Effect of increased sodium chloride intake on blood pressure.* Six hypertensive patients, 4 of whom had been included in the previous study, were investigated following a preliminary baseline period and constant regimen which included 4 grams of sodium chloride added to the salt-poor diet. The intake of sodium chloride was then increased to 15 grams daily for an 8-day period. These subjects were able to tolerate this dosage without gastric intolerance or diarrhea.

A rise in *"resting"* blood pressure was observed in 5 patients within 1 to 4 days after the addition of sodium chloride to the diet, with a return to previous levels after the initial dosage was resumed (Figure 2, II). (In 2 patients a week's trial with placebo pills was without effect on the blood pressure.) Again *"casual"* readings were uninfluenced and each patient exhibited the same variation, the same response to such stimuli as activity, apprehension or the cold pressor test as was demonstrated before the increased sodium chloride intake.

The further addition of sodium chloride for a period of 8 days was followed by an increase in urine sodium and chloride excretion, and slight weight gain and decreases in urine output were generally observed. No significant change was recorded in serum CO_2, chlorides, sodium, potassium, protein, urea nitrogen, or volume measurements (Table II).

III. *Effect of rigid sodium chloride restriction*

TABLE I

Clinical and laboratory data of 6 hypertensive patients before and after rigid sodium chloride restriction

Case	Mean "resting" B.P. — week before NaCl restriction	Mean "resting" B.P. — 2nd week of NaCl restriction	Mean "casual" B.P. — week before NaCl restriction	Mean "casual" B.P. — 2nd week of NaCl restriction	B.P. response to cold pressor test — before	B.P. response to cold pressor test — after	Before and after 2 weeks of rigid NaCl restriction: Urine* Sodium	Urine* Chloride	Serum CO₂ content	Serum Chloride	Serum Sodium	Serum Potassium	Serum Proteins	Serum Urea nitrogen	Serum Volume
							m.eq. per 24 hrs.	*m.eq. per 24 hrs. as NaCl*	*m.eq. per liter*	*m.eq. per liter*	*m.eq. per liter*	*m.eq. per liter*	*grams per 100 cc.*	*mgm. per 100 cc.*	*cc.*
1	164/106	148/92	182/114	180/112	196/116	200/120	86– 9	88–11	26.4–29.6	104.1–103.1	139.1–140.5	4.3–4.2	6.4–6.6	13–16	2460–2420
2	168/110	152/96	178/116	180/116	194/122	190/120	82–13	94–14	29.0–28.6	102.4–100.3	138.4–139.3	4.2–4.0	6.1–7.0	8–14	2710–2680
3	182/104	160/92	—	—	—	—	68–13	90–16	27.7–24.2	104.6–104.1	142.5–135.7	4.4–5.4	6.7–6.9	7– 9	
4	160/98	140/90	174/104	170/100	182/110	188/112	69– 8	64–20	33.5–30.1	97.5– 99.0	140.1–139.8	4.4–4.8	6.7–6.8	12–14	2360–2280
5	162/110	146/100	176/120	172/118	—	—	68– 9	70–14	27.9–29.7	100.4– 98.1	138.5–138.5	4.5–4.5	7.3–7.2	14–18	
6	182/116	168/108	204/110	208/114	216/120	206/118	66–14	76–15	27.3–28.5	100.8– 99.2	137.5–137.7	4.4–4.4	7.2–7.4	11–10	2000–2000

* Mean values for each period.

on pressor action of DCA (12). Five hypertensive patients, 3 of whom had been investigated in the previous studies, were studied following a preliminary baseline period and constant regimen which included 4 grams of sodium chloride added to the salt-poor diet. DCA was then injected subcutaneously in doses of 5 mgm. twice daily, with added dietary sodium chloride increased in 3 subjects but maintained at 4 grams in 2. After 10 days, all added sodium chloride was withdrawn from the diet without other change in regimen and while the subjects continued to receive DCA.

In all instances an increase in *"resting"* blood pressure followed the injection of DCA, this rise not being maintained when sodium chloride was withdrawn (Figure 3). During the period of sodium chloride and DCA administration, the expected slight increases in weight, hemodilution and serum volume were generally noted, together with slight reduction in urine volume (12, 13). Significant increases in serum chloride and sodium were observed in 2 patients, while reductions in serum potassium of from 0.4 to 1.5 m.eq. took place in all subjects. When DCA injection was continued but with rigid sodium chloride restriction, slight reductions in weight and serum volume were seen, together with hemoconcentration, slight increases in urine volume, and a sharp decrease in urine sodium and chloride values. The serum chloride and sodium were significantly reduced by sodium chloride restriction in 2 instances, while the serum potassium levels showed

TABLE II

Clinical and laboratory data of 6 hypertensive patients before and after increased sodium chloride intake

Case	Mean "resting" B.P.		Mean "casual" B.P.		B.P. response to cold pressor test		Before and after 8 days of increased NaCl intake								
							Urine*		Serum						
	week before	*8 days of*	*week before*	*8 days of*	*before*	*after*	Sodium	Chloride	CO_2 content	Chloride	Sodium	Potassium	Proteins	Urea nitrogen	Volume
	Increased NaCl intake						*m.eq. per 24 hrs.*	*m.eq. per 24 hrs. as NaCl*	*m.eq. per liter*	*m.eq. per liter*	*m.eq. per liter*	*m.eq. per liter*	*grams per 100 cc.*	*mgm. per 100 cc.*	*cc.*
1	140/96	158/108	160/110	160/112	178/120	174/118	57–190	74–176	31.4–32.8	99.4– 99.0	140.0–140.8	4.4–4.2	6.4–6.4	13–11	2440–2500
2	166/100	180/112	210/120	206/120	220/120	220/120	72–204	80–200	27.4–28.0	100.4– 99.6	138.4–138.4	4.9–4.6	6.0–5.9	18–18	2620–2580
3	162/112	164/116	176/122	174/120	—	—	66–210	74–210	26.3–27.0	101.9–101.9	138.1–138.9	4.6–4.9	7.0–7.2	14–17	
4	146/92	160/102	162/104	162/100	180/118	182/118		90–216							2480–2520
5	162/102	178/110	186/114	188/114	196/118	200/120	86–204	68–178	29.9–30.4	104.4–103.3	140.9–138.0	4.9–4.9	6.2–6.0	14–16	2840–2920
6	172/102	184/110	196/116	194/118	—	—	65–186	74–230	24.4–26.1	106.3–108.0	140.7–140.0	5.2–4.7	6.1–6.0	9–13	

* Mean values for each period.

no further consistent change (Figure 4, Table III).

As the pressor action of prolonged DCA injection was on occasion found to be transitory, the order was reversed in 2 additional patients, *i.e.*, following the usual baseline observations, rigid sodium chloride restriction was instituted simultaneously with the start of DCA administration. These were patients who had not been subjected to previous studies. No rise in *"resting"* blood pressure ensued until the addition of sodium chloride (Figure 5).

COMMENT

The present study, utilizing measurements of the *"resting"* blood pressure, confirms the reports of some previous observers that the rigid restriction of sodium chloride in the diet may lower the blood pressure of patients with hypertensive vascular disease. Conversely, it has been shown under similar conditions that large amounts of dietary sodium chloride may exert a pressor effect in hypertensive subjects. Lastly, the observations of Knowlton and her associates (11), that sodium chloride participates in the potentiation of the pressor activity of DCA, have been supported by studies in patients with an elevated blood pressure. It has been previously shown that this pressor response to DCA is not due to salt and water retention alone (12).

Because of the great variability of blood pressure readings in association with any of a large

number of environmental changes, the need has been stressed for an adequate baseline and constant regimen as well as for the differentiation between *"resting"* and *"casual"* values. It must be emphasized that the magnitude of blood pressure fluctuation observed in this study was often small, although consistent, and that standard conditions were required.

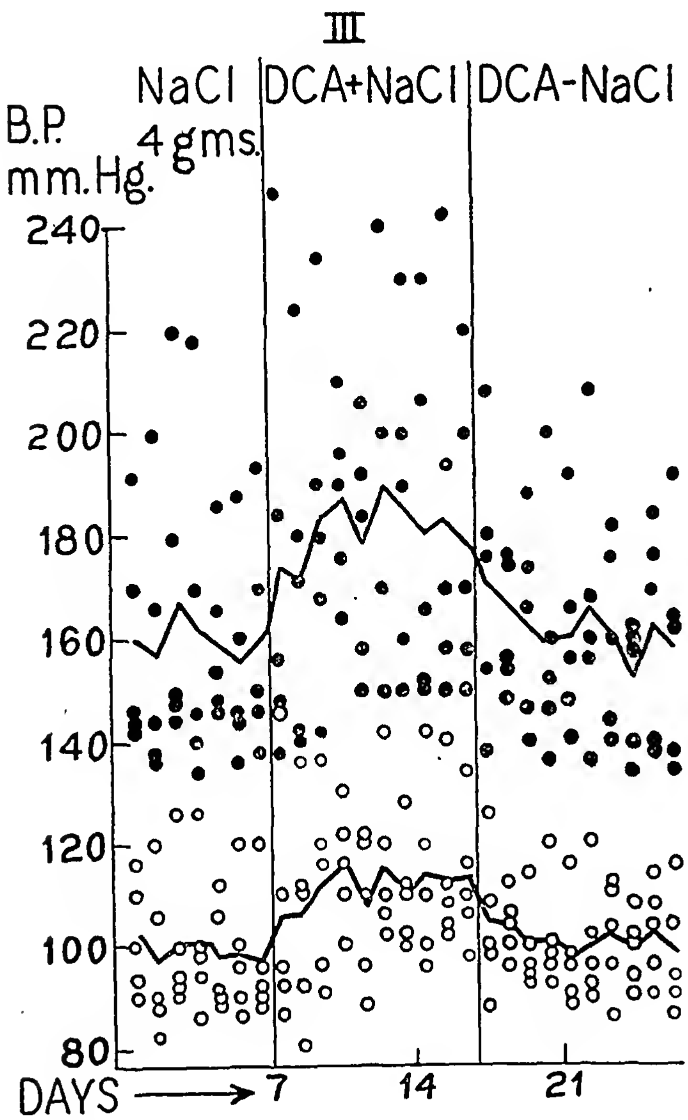


FIG. 3. EFFECT OF RIGID SODIUM CHLORIDE RESTRICTION ON PRESSOR ACTION OF DCA IN 5 HYPERTENSIVE SUBJECTS

The effect of rigid sodium chloride restriction is apparently unrelated to changes in circulatory volume or cardiac output, hence must be concerned with alterations in peripheral resistance. It must be noted that a normal blood pressure is not routinely achieved by salt restriction and that the patient's ability to respond to autonomic or neurogenic stimuli is unaffected. The demonstration in this study that there is an absence of correlation between *"resting"* and *"casual"* blood pressure readings, suggests that the factors (at least in part neurogenic) influencing the "casual" readings may be independent of those influenced by sodium chloride or DCA. This may explain the results of sympathicolytic drugs and of surgical procedures which modify vasoconstriction of sympathetic origin yet do not invariably relieve the hypertensive state. Previous uncertainties concerning the effect of many therapeutic agents may have been due to the failure to dissociate between extrinsic (neurogenic) and direct actions on peripheral resistance, both of which seem to be involved—in variable proportions—in the clinical picture of hypertension.

There is nothing to suggest that the small changes in *"resting"* blood pressure associated with rigid sodium chloride withdrawal are of therapeutic significance or that prolonged restriction will exert any influence on the natural history of this disorder.

The modification of *"resting"* blood pressure levels by sodium chloride and the relationship of sodium chloride to the pressor action of DCA are consistent with the view that some product of the adrenal cortex may play a part in the mechanism of hypertensive vascular disease (12, 14, 15).

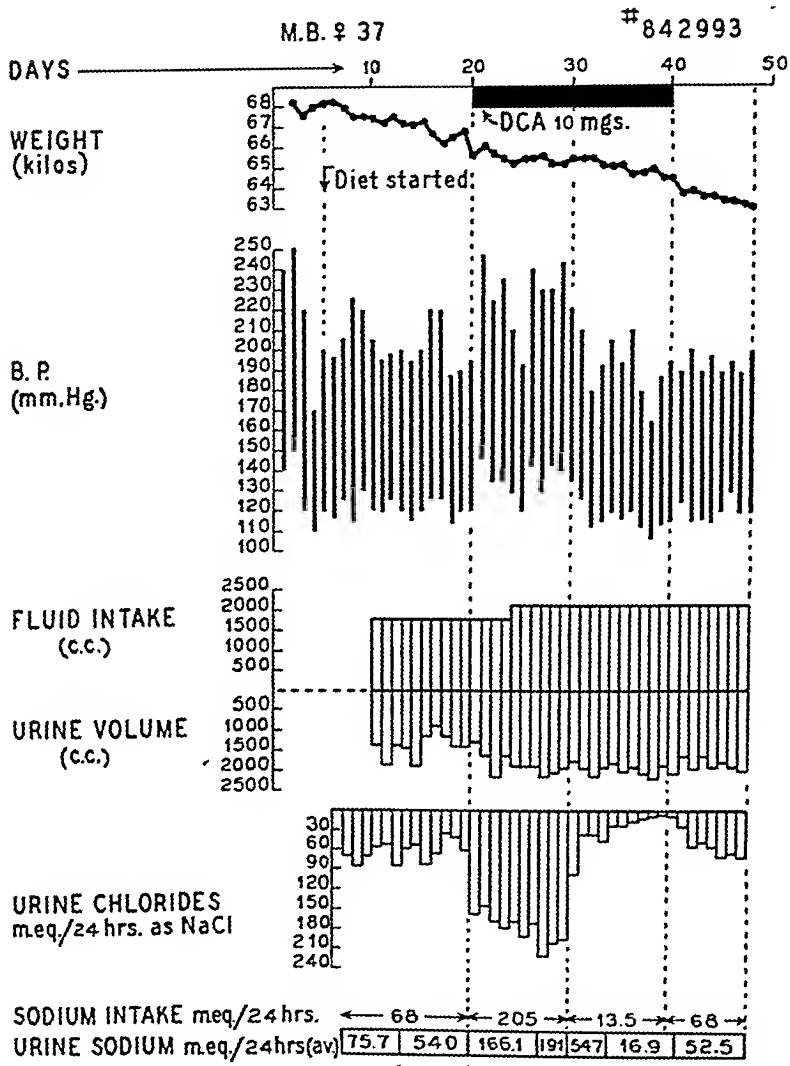
CONCLUSIONS

1. Observations have been made on patients with uncomplicated hypertensive vascular disease under controlled conditions of diet and activity.

2. Despite an otherwise constant regimen, rigid sodium chloride restriction resulted in a slight decrease in *"resting"* blood pressure in 6 subjects, whereas large amounts of dietary sodium chloride produced a slight rise in *"resting"* blood pressure in 5 of 6 subjects.

3. The pressor action of desoxycorticosterone acetate was observed in 7 subjects, but only when sodium chloride was included in the diet; the rigid restriction of sodium chloride obliterated this pressor response.

4. It is suggested that vasoconstrictive factors (at least in part neurogenic) in hypertensive patients are independent of those alterations in peripheral resistance influenced by sodium chloride or desoxycorticosterone acetate.



SERUM CO_2 CONTENT m.eq./liter	35.7	34.0	35.2	39.3	39.8	35.4
SERUM CHLORIDES m.eq./liter	92.0	92.4	97.8	91.0	89.4	96.0
SERUM SODIUM m.eq./liter	142.4	141.4	144.0	141.4	141.2	144.1
SERUM POTASSIUM m.eq./liter	3.3	3.3	3.1	2.3	2.7	2.5
SERUM PROTEIN Gms/100 c.c.	6.9	6.5	6.6	6.6	6.5	6.4
SERUM UREA NITROGEN mgs./100 c.c.	15	12	14	12	14	12

Fig. 4. Clinical and Laboratory Data Illustrating Effect of Rigid Sodium Chloride Restriction on Pressor Action of DCA

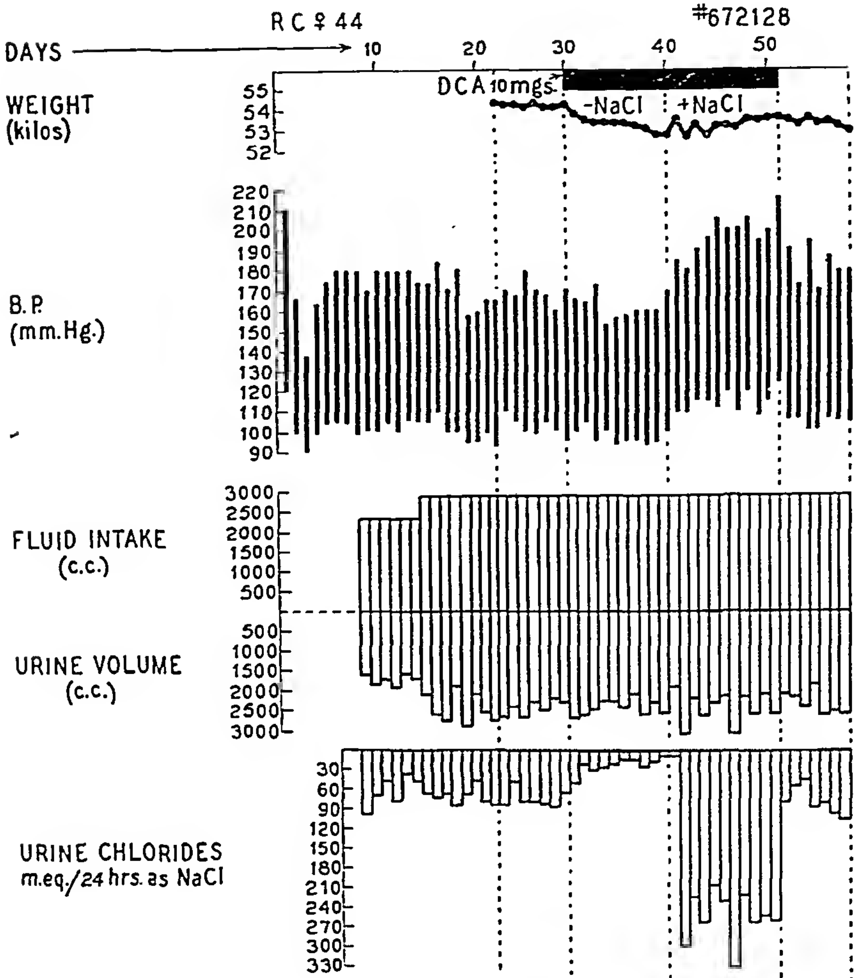
TABLE III

Laboratory data of 5 hypertensive patients—effect of rigid sodium chloride restriction on pressor action of DCA

Case	Mean "resting" B.P.			Urine† Sodium			Urine† Chloride			Serum CO₂ content			Serum Chloride			Serum Sodium			Serum Potassium			Serum Proteins			Serum Urea nitrogen			Serum Volume		
	I*	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
				m. eq./24 hrs.			*m. eq./24 hrs. as NaCl*			*m. eq./liter*			*m. eq./liter*			*m. eq./liter*			*m. eq./liter*			*grams/100 cc.*			*mgm./100 cc.*			*cc.*		
1	200/118	226/120	198/110	57	133	29	62	182	32	27.6	30.6	32.8	101.5	101.3	97.2	138.0	140.0	139.0	4.7	3.2	4.0	7.2	5.8	6.8	12	9	14	2940	3000	—
2	166/120	200/136	174/118	54	166	22	58	179	25	34.0	35.2	39.8	92.4	97.8	89.4	141.4	144	141.2	3.3	2.6	2.7	6.5	6.6	6.5	12	13	14		—	
3	146/98	172/110	156/104	72	58	12	82	70	18	23.7	30.0	31.7	104.6	102.8	98.0	135.8	139.2	140.4	4.3	3.4	3.5	7.0	6.7	7.3	13	11	12	2000	2290	1705
4	142/94	160/100	146/92	85	78	18	92	84	28		—		103.8	107.1	104.2	137.2	137.4	136.9	4.8	4.4	4.4	6.4	6.0	6.2		—		2640	2860	2720
5	144/90	152/100	140/90	65	210	15	78	246	27	25.6	30.0	28.4	104.1	98.6	101.2	136.0	139.8	135.4	4.9	3.9	3.6	6.6	6.4	6.8	10	8	12	2400	2580	2420

* I. Preliminary week—NaCl 4 grams added to diet.
II. Ten-day period—DCA 10 mgm. daily, NaCl 4 to 15 grams added to diet.
III. Ten-day period—DCA 10 mgm. daily, rigid NaCl restriction.

† Mean values for each period.



SODIUM INTAKE m.eq./24 hrs. ← 68. → ← 13.5 → ← 25 6. → ← 68. →

URINE SODIUM m.eq./24 hrs. 58.1 | 65.2 | 77.6 | 15.43 | 102.6 | 231.9 | 98.4

SERUM CO_2 CONTENT m.eq./l.	25.7	26.2	24.7	24.3	27.1	28.7	30.4	27.7
SERUM CHLORIDES meq./liter	105.6	107.0	101.4	105.2	102.5	102.5	99.4	104.6
SERUM SODIUM m.eq./liter	141.3	143.2	136.6	136.2	135.5	136.2	139.5	142.5
SERUM POTASSIUM meq./liter	4.4	4.8	4.1	4.8	4.0	3.7	2.9	4.4
SERUM PROTEIN Gms./100 c.c.	6.8	6.3	6.9	6.6	6.3	6.9	6.8	6.7
SERUM UREA NITROGEN mgs./100 c.c.	13	14	11	10	12	9	8	7

FIG. 5. CLINICAL AND LABORATORY DATA ILLUSTRATING EFFECT OF RIGID SODIUM CHLORIDE RESTRICTION ON PRESSOR ACTION OF DCA

5. The relationship of sodium chloride and desoxycorticosterone acetate to hypertensive vascular disease is consistent with the view that some product of the adrenal cortex may play a part in the mechanism of this disorder.

BIBLIOGRAPHY

1. Volhard, F., Die Behandlung der Sklerosen, in von Bergmann, G., and Staehelin, R., Handbuch der inneren Medizin, Berlin, Julius Springer, Vol. 6, Pt. 2, p. 1753, 1931, Ed. 2.
2. Ambard L., and Beaujard, E., La rétention chlorurée sèche. Semaine méd., 1905, 25, 133.
3. Allen, F. M., Arterial hypertension. J. A. M. A., 1920, 74, 652.
4. Mosenthal, H. O., The treatment of high blood pressure. M. Clinics N. America, 1922, 5, 1139.
5. McLester, J. S., The influence of rigid salt restriction in the diet of chronic nephritis. Am. J. M. Sc., 1922, 163, 794.
6. O'Hare, J. P., and Walker, W. G., Salt in vascular hypertension. Arch. Int. Med., 1923, 32, 283.
7. Berger, S. S., and Fineberg, M. H., The effect of sodium chloride on hypertension. Arch. Int. Med., 1929, 44, 531.
8. Selye, H., Hall, C. E., and Rowley, E. M., Malignant hypertension produced by treatment with desoxycorticosterone acetate and sodium chloride. Canad. M. A. J., 1943, 49, 88.
9. Grollman, A., Harrison, T. R., Mason, M. F., Baxter, J., Crampton, J., and Reichsman, F., Sodium restriction in the diet for hypertension. J. A. M. A., 1945, 129, 533.
10. Kempner, W., Treatment of kidney disease and hypertensive vascular disease with rice diet. North Carolina M. J., 1944, 5, 125.
11. Knowlton, A. I., Loeb, E. N., Stoerck, H., and Seegal B. C., Desoxycorticosterone acetate: the potentiation of its activity by sodium chloride. J. Exp. Med., 1947, 85, 187.
12. Perera, G. A., and Blood, D. W., The pressor activity of desoxycorticosterone acetate in normotensive and hypertensive subjects. Ann. Int. Med., 1947, 27, 401.
13. Clinton, M., Jr., and Thorn, G. W., Effect of desoxycorticosterone acetate administration on plasma volume and electrolyte balance of normal human subjects. Bull. Johns Hopkins Hosp., 1943, 72, 255.
14. Perera, G. A., The relationship of the adrenal cortex to hypertension: observations on the effect of hypoadrenalism on a patient with hypertensive vascular disease. J. A. M. A., 1945, 129, 537.
15. Perera, G. A., and Blood, D. W., Disturbance in salt and water metabolism in hypertension. Am. J. Med., 1946, 1, 602.

CAPILLARY PERMEABILITY IN RELATION TO ACUTE ANOXIA AND TO VENOUS OXYGEN SATURATION[1]

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INTRODUCTION

It has often been assumed that moderate degrees of hypoxia render the capillaries abnormally permeable to fluid and protein. During the course of work concerned with methods of insuring pilot survival at altitudes above 45,000 ft., it became desirable to know the degree and duration of anoxic anoxia that could be tolerated before permeability changes of the capillary bed occurred. The studies of Landis (1) have demonstrated that complete ischemic anoxia of 3 minutes' duration will cause temporary damage to the capillaries of the frog's mesentery and result in abnormal permeability to fluid and protein. Pochin (2) working with the rabbit's ear has found that complete occlusion of the circulation for 2 hours will lead to demonstrable edema following re-establishment of the circulation. He also showed that 16 to 18 hours of occlusion resulted in edema fluid containing approximately 5 grams per cent of protein. Calvin (3) has found that during the terminal stages of fatal asphyxia there is evidence of a marked loss of protein and of the protein-attached dye T 1824 from the capillary bed.

These experiments involved very intense anoxia and on turning to work done under less severe conditions the results are seen to be less conclusive. Thus Calvin (3) and also Hopps and Lewis (4) found no change in the rate of filtration of plasma protein and of the dye T 1824 in the course of severe but non-fatal anoxia. On the other hand Maurer (5) and Warren and Drinker (6) have shown that, in the dog, the flow of cervical and lung lymph increases during severe but non-fatal anoxic anoxia. However the percentage of protein in this lymph decreases with the increased flow and the results do not unequivocally demonstrate an increase in capillary permeability as a result of the acute anoxia. Observations concerning the influence of anoxia have been made in humans. McMichael and Morris (7) have used nitrogen-oxygen gas mixtures of an oxygen percentage (9.5 per cent) that would induce approximately 65 per cent arterial hemoglobin saturation. They found no increase in the rate of swelling of the congested arm. Their results suggested that the permeability of the arm capillaries to fluid is unchanged by anoxia. However, the technique employed did not give them any information on the concentration of the protein in the filtrate and their report is in abstract form only. Another study is that by Stead and Warren (8) who have estimated the protein content of the edema fluid in 2 emphysematous patients whose arterial oxygen saturation was 50 to 60 per cent. They found no significant change from the normal figure of 0.25 gram per cent or less and concluded that this degree of chronic anoxia had not increased the permeability of the capillaries of the limbs. In an earlier report (9) the authors found no significant change in fluid or protein loss from the capillaries of the forearm when under the influence of acute anoxia.[2] The following paper confirms these results and extends them to an estimation of the approximate oxygen tension at which a significant increase in protein filtration from the capillary bed occurs.

[1] This work was supported in part by the AAF Aero Medical Laboratory, Air Materiel Command, Wright Field.

[2] A systematic error which does not however invalidate the conclusions drawn has been detected in the calculation of the protein data presented in this report to the CAM of the OSRD and summarized in the Fed. Proc., 1946, 5, 44. The corrected figures for the calculated protein in the filtrate are 1.5 grams ± 1.5 grams per cent when anoxic and 1.2 grams ± 0.5 gram per cent when at sea level.

METHOD OF APPROACH

The best measure of the permeability of the capillary wall would be a direct determination of the concentration of protein in the capillary filtrate (10). A significant increase in this concentration over the normal near zero (0.2 to 0.3 gram per cent) values found in the limb may be regarded as evidence of a change in the permeability of the wall (1, 8). Failing a direct determination an estimate of this measure was made by Landis *et al* (11) in their experiments, using various degrees of venous congestion supplied by the blood pressure cuff. The congestion cuff causes a marked slowing of blood flow without stopping it when the venous pressure imposed by it (60 or 80 mm. Hg) is less than the arterial blood pressure. The increased filtration pressure of the slowly flowing blood results in a loss of fluid into the tissues. Comparison of the hematocrit values shown by the blood in the median antecubital vein of the congested arm with that in the uncongested control arm permits an estimation of the fluid loss in ml. per 100 ml. of the blood flowing through the congested arm. These investigators have devised a formula by which they can calculate the percentage of protein in the capillary filtrate from observations on the relative changes in hematocrit values and plasma protein levels in the blood from the control and congested arms. In such studies on the human arm, they have observed a calculated protein in the capillary filtrate at a congestion pressure of 80 mm. Hg of approximately 1.5 grams per cent. This procedure produces the double effect of an increase of the hydrostatic pressure in the capillaries and an ischemic anoxia due to slowing of the blood flow through the vessels. It has been pointed out (11) that if it were possible to be sure that the high pressure in the congested capillary bed was not directly responsible for this abnormally high content of protein in the filtrate, then the experiment would provide evidence of the effect of ischemic anoxia on capillary permeability. Kunkel, Stead and Weiss (12), and more recently Allen *et al* (13) have shown that adrenalin injections will lead to a marked increase in the amount of blood flowing through a limb. If this increase in blood flow is enough to effectively eliminate the ischemic anoxia resulting from a congestive cuff then it should be possible to eliminate the protein loss in a congested arm by increasing the blood flow through it by the use of adrenalin. If the protein loss is eliminated in spite of the use of cuff pressures of 80 mm. Hg then it can be assumed that such pressures have little effect on the permeability of the blood vessels. Another method of dissociating the effects of pressure from those of anoxia would be to decrease the venous oxygen tension while keeping the capillary pressure constant. This could be accomplished by exposing the subject to a lowered alveolar oxygen tension at a cuff pressure at which the protein in the filtrate was at the upper limit of the normal values. Reduction of the arterial oxygen saturation to 60 to 70 per cent would cause a considerable fall in the mean capillary oxygen tension. This fall should increase the protein in the capillary filtrate if permeability is significantly affected by changes in oxygen tension. Since the oxygen tension is lowest at the venous end of the capillary, the oxygen content of the venous blood should be a measure of the oxygen tension in that part of the capillary bed which is exposed to the most severe anoxia. If the content of protein in the filtrate is a measure of the permeability of the capillary wall and if this permeability is affected by the oxgen tension, then it should be possible to demonstrate a correlation between the oxygen content of the venous blood from the congested arm and the concentration of protein in the capillary filtrate.

METHODS

The subjects employed were chosen for their familiarity with experimental procedures and the majority of the tests were performed on only 3 persons. These were in good health and of an active habitus. The 2 subjects employed for the altitude studies showed no gross vasomotor disturbances with acute anoxic anoxia. There was no significant change in blood pressure, pulse or respiration rate with reduction of the arterial oxygen saturation to values of the order of 60 per cent for periods of 30 minutes. It was hoped that by employing repeated tests on a few well-trained subjects it would be possible to decrease the inherent differences between various subjects and the variations due to fluctuations of subject response.

Adrenalin hypertension was obtained by the intramuscular administration to the recumbent subjects of 6/10 ml. of 1/500 adrenalin in peanut oil followed in 15 minutes by 1/10 ml. doses of 1/1000 adrenalin subcutane-

ously in sufficient number to maintain the blood pressure 20 to 40 mm. Hg above the normal resting level for the subject. In the subjects employed this meant that the systolic blood pressure was maintained at 140 to 160 mm. Hg for the 30 minutes of 80 mm. Hg cuff application.

During the altitude tests each subject rested for 30 minutes before putting on the cuff at 60 mm. Hg for 30 minutes. The temperature of the chamber in which the tests were conducted was uniform. However, no readings of skin temperature were made and it is thought that some of the variations in the results noted may have been due to minor changes in the condition of the skin and deep circulation from day to day as the tests progressed. The same arm was always used for congestion in order to decrease experimental variations.

Bloods were collected with 0.1 ml. of liquid heparin in 10-ml. syringes and immediately set up in duplicate in Wintrobe hematocrit tubes. Duplicate hemoglobins were estimated using the acid hematin technique. R.B.C. volume was obtained by correcting the hematocrit readings by a factor of 8.5 per cent for fluid trapped between the cells (14). Plasma protein concentrations were determined in duplicate using the falling drop technique. Venous oxygen content and saturations together with venous carbon dioxide contents were determined in duplicate by Van Slyke blood gas analysis in a number of the tests using 60 mm. Hg congestion and anoxia and in all those using 80 mm. Hg congestion with adrenalin hypertension.

Anoxia involving 55 to 65 per cent saturation of the arterial blood was obtained by ascent in a decompression chamber to a simulated altitude of 19,000 to 20,000 ft. The subjects were relaxed and dozed in a recumbent posture on a couch. Respiratory rate, blood pressure and pulse were checked every 5 minutes and the oxygen saturation followed throughout by a Millikan compensated oximeter. Contamination of the altitude chamber atmosphere by oxygen was avoided by using an oxygen mask with an exhalation valve which was connected to a tube venting directly into the chamber exhaust. The oxygen saturation, rarely higher than 65 per cent, was maintained at 60 per cent and frequently fell to 55 per cent.

Calculations

The equations used to determine the per cent protein in the filtrate were those developed by Landis *et al* (10). As they have pointed out (10, 11) these equations become less accurate with lower degrees of fluid and protein loss. In order to assess the variability introduced by these calculations of protein in the filtrate, the standard errors of the hematocrit and protein readings were determined. These were found by making repeated measurements of the same sample. They were ± 0.2 per cent for the hematocrits and ± 0.06 gram per cent for the plasma proteins. These values were inserted into the equations and the maximum positive and negative deviations were calculated for each set of experimental data. In Figure 1 such values obtained for each experiment have been plotted against the product of the hematocrit and protein differences, yielding a parameter which indicates in part at least the variability to be anticipated in the protein in the filtrate. If the product $(Ht_2 - Ht_1)(Pr_2 - Pr_1)$ is small, a condition which would be associated either with a low fluid loss and usually small protein leakage or both, then the variability is great. High values show an increased accuracy. Inspection of Figure 1 also reveals a negative bias which is largest at the lowest values of protein change and fluid loss.

In a considerable proportion of the experiments the calculations yield negative values for protein concentration in the filtrate. Such negative values might result from a failure to take account of unrecognised physiological variables. Alternatively, in view of the large random error and more especially of the negative bias introduced by the equation, the values obtained could be the result of the mathematical skewing of the formula with consequent negative bias noted above.

RESULTS

1. Adrenalin hypertension. In Table I are presented the results of 8 cuff experiments on 3

TABLE I

Fluid loss and per cent protein in filtrate with 80 mm. Hg congestion (controls)

Subject	Hematocrit	Fluid loss (hematocrit)	Fluid loss (hemoglobin)	Protein	Protein in filtrate	Saturation of venous blood
		ml. per 100 ml.		*grams*	*per cent*	
5c	43.4 45.8	5.6	3.9	6.55 7.10	+1.5	
5d	41.9 46.9	10.9	8.6	6.19 7.37	+1.0	21.6
5e	40.7 46.1	11.7	10.7	5.76 6.93	+1.0	21.3
7q	41.3 47.2	12.6	18.2	6.76 7.79	+3.0	18.9
7r	42.8 52.7	18.9	19.1	6.11 8.88	+0.5	19.1
7s	43.0 52.6	18.3	17.5	5.90 8.66	+0.1	15.6
6j	39.5 43.5	9.4	6.6	5.97 7.27	−1.1	18.2
6k	39.6 49.6	20.1	17.0	6.14 7.82	+2.8	16.9
6l	41.0 47.2	13.2	13.9	5.80 7.92	−1.6	12.1
Average Standard deviation of mean		13.4 ±1.7	12.8 ±1.8		+0.8 ±0.5	18.0

subjects using a cuff at 80 mm. Hg for 30 minutes. There is fair agreement between the mean values for the fluid loss as calculated from the changes in the hematocrit, 13.4 ± 1.7 ml. per 100 ml., and those calculated from the hemoglobin values, *i.e.*, 12.8 ± 1.8 ml. per 100 ml. This suggests that no significant changes in cell volume occur as a result of the stasis induced by the cuff experiment, for such changes would affect only the hematocrit values (10). The marked individual variations from experiment to experiment may be in part attributed to changes in rate of forearm blood flow due to differences in temperature, activity and other variables. Attempts to obtain more reliable figures by using an indwelling needle in the radial artery (15) as a source of the control blood led to no improvement. However, it is of interest that in their original series of 5 experiments using 80 mm. Hg cuff pressure, Landis, *et al* obtained a mean fluid loss figure of 15.0 ml. per 100 ml. This compares favorably with the above values.

In Table II are seen the data obtained from the same number of experiments on the same 3 subjects performed in the same way except that a hypertension of 140 to 160 mm. Hg was maintained throughout the ½-hour period of cuff application. The fluid loss figures show good concordance between the hemoglobin and hematocrit values. The values of 6.0 ± 0.6 and 6.6 ± 0.8 ml. per 100 ml., respectively, are almost exactly ½ those obtained for the normal controls. This is interesting in view of the observation by Allen *et al* (13) that adrenalin administration can double blood flow through the uncongested arm. For if the blood flow rate were doubled and other factors such as tissue pressure remained relatively unchanged then the fluid loss per 100 ml. blood should be decreased in the same proportion.

It might be expected that the A-V O_2 difference would be greatly decreased if such a change in flow rate had occurred. In practice the mean venous oxygen saturation rose from 18.0 per cent to 30.5 per cent. It is of interest that numbness, tingling and eventual loss of sensation to light touch developed in the congested arms in the controls. Such symptoms never occurred at 80 mm. Hg cuff pressure in the cases with adrenalin hypertension. This suggests that the blood flow under these conditions is adequate to maintain normal metabolism but that the reduced flow in the absence of hypertension is insufficient to do so. It may be noted that Griffith, *et al* (16) point out that it is probable that adrenalin has no significant effect on tissue oxygen utilization. Therefore the rise in oxygen saturation following its use is probably not due to a decrease in tissue metabolism.

The change in the average protein percentage in the filtrate from + 0.8 ± 0.5 gram per cent to a mean value of − 1.0 ± 0.3 grams per cent as a result of the use of adrenalin is significant. It is not probable that the percentage of protein in the filtrate would change with changes in the rate of flow of the plasma past the filtering area. It would seem likely that the increase in mean venous oxygen saturation has had some part to play in the decrease in protein in the filtrate. The negative mean value for the protein figures can be explained by the bias of the expression for protein in the filtrate to negative values by the random errors of sampling and estimation. As has been

TABLE II

Fluid loss and per cent protein in filtrate with 80 mm. Hg congestion (adrenalin hypertension)

Subject	Hematocrit	Fluid loss (hematocrit)	Fluid loss (hemoglobin)	Protein	Protein in filtrate	Saturation of venous blood
		ml. per 100 ml.		*grams*	*per cent*	
5f	40.1 42.1	4.9	3.9	5.42 6.17	−3.0	24.3
5g	40.0 44.0	9.2	10.4	5.97 7.07	−0.1	27.8
7t	44.2 47.4	6.8	4.8	6.17 7.24	−1.5	42.3
7u	44.2 48.3	8.5	7.0	6.35 7.58	−0.5	35.4
7v	44.8 46.5	3.6	4.9	6.52 7.03	−0.8	51.9
6m	42.8 45.2	5.1	5.2	6.48 7.03	+0.9	19.5
6n	43.5 47.2	8.1	7.9	6.93 8.23	−0.8	14.3
6o	42.5 46.2	7.9	8.9	5.93 7.27	−2.5	28.4
Average Standard deviation of mean		6.0 ±0.6	6.6 ±0.8		−1.0 ±0.3	30.5

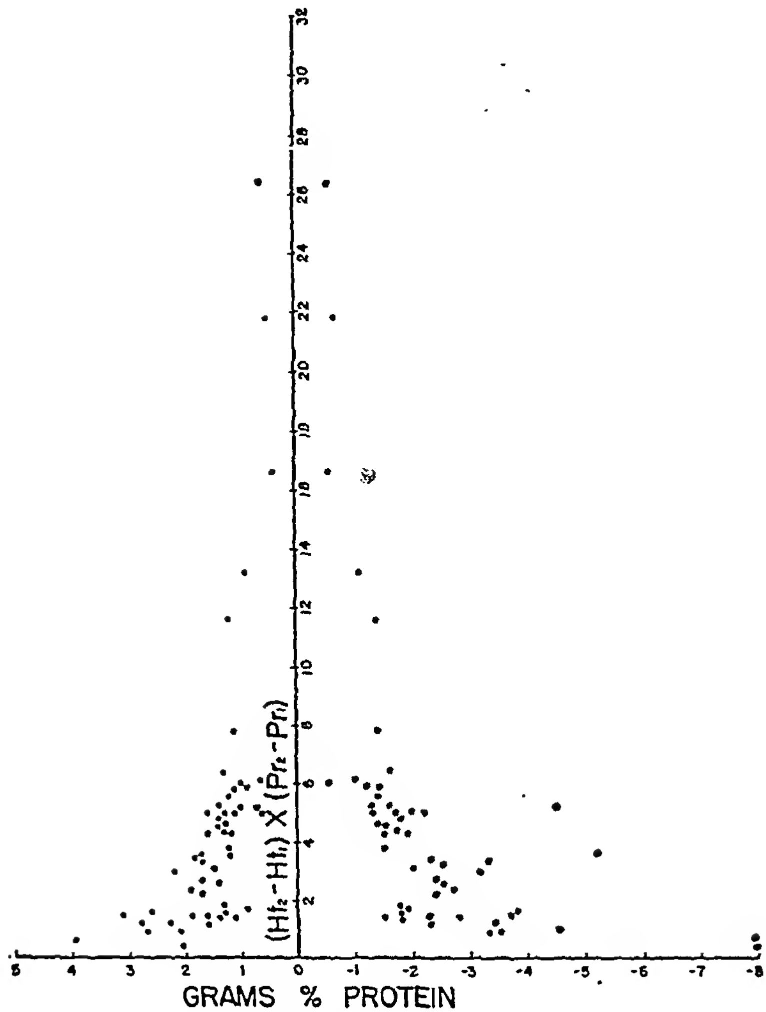

FIG. 1. DEVIATION IN GRAMS PER CENT PROTEIN OF THE PROTEIN IN THE FILTRATE ESTIMATIONS PLOTTED AGAINST THE PRODUCT OF THE CHANGES IN THE HEMATOCRIT ($HT_2 - HT_1$) AND THE PROTEIN VALUES ($PR_2 - PR_1$)

pointed out under calculations (Figure 1) this error is greater in the case of the adrenalin experiments in which the fluid loss is small than in those where these changes are larger, as in the control experiments. It is not certain, however, whether there may not also be some undetermined variable which may account in part for the high negative values found in the adrenalin experiments.

The object of the adrenalin tests was to decrease the ischemic anoxia while keeping the capillary pressure constant. Since the protein in the filtrate decreased under these circumstances the possibility that the high intracapillary pressure was responsible for the increased filtration of protein in the 80 mm. Hg cuff without adrenalin can be considered small. This leaves ischemic anoxia as the most probable factor responsible for the permeability changes.

2. *Anoxia.* In Tables III and IV are presented a series of 23 control, sea level, 60 mm. Hg cuffs on 7 subjects, and 12 altitude experiments using 2 subjects. The mean sea level fluid loss of 7.6 ml. per 100 ml. ± 0.5 ml., as calculated from the hematocrit values, was again close enough to that calculated from the hemoglobins

TABLE III

Fluid loss and per cent protein in filtrate with 60 mm. Hg congestion (controls)

Subject	Hematocrit	Fluid loss (hematocrit)	Fluid loss (hemoglobin)	Protein	Protein in filtrate	Saturation of venous blood
		ml. per 100 ml.		*grams*	*per cent*	
1a	40.4/43.2	5.9	8.5	7.05/8.10	−2.5	
1b	41.4/43.8	4.5	5.1	7.30/8.03	−1.5	
1c	40.7/42.5	4.3	1.5	6.88/7.23	+2.5	
2a	38.6/42.4	9.1	6.7	6.94/8.10	+0.3	
2b	39.3/43.0	8.7	7.4	6.68/7.75	+0.3	
3a	38.3/41.2	7.1	4.0	6.50/7.03	+2.4	
3b	41.7/43.4	3.6	3.0	6.90/7.30	+0.8	
4a	43.0/45.7	5.8		6.46/7.28	−0.7	
5a	42.8/45.9	6.8	2.0	6.04/6.79	+0.5	28.7
5b	41.0/43.0	4.7	6.3	5.52/6.11	−1.3	33.5
6a	41.5/47.1	12.0	12.1	6.48/7.31	+3.3	13.3
6b	40.9/43.6	6.1	9.6	6.17/6.86	+0.2	17.2
6c	41.2/45.7	10.0	9.4	6.04/6.83	+2.2	23.0
7a	42.5/46.7	8.8	13.9	6.27/7.28	+0.6	
7b	42.1/47.0	10.5	12.4	6.53/7.75	+1.0	
7c	44.2/48.2	8.4	3.1	6.45/7.61	−0.1	
7d	46.2/49.8	7.3		6.14/7.34	−1.5	
7e	46.0/48.6	5.3	5.1	7.00/7.51	+2.3	
7f	41.5/44.7	7.4	6.9	6.07/7.03	−0.6	
7g	42.4/47.1	10.1	20.3	6.55/7.65	+1.4	
7h	41.4/46.3	10.7	12.6	5.83/7.44	−1.4	25.7
7i	42.5/47.1	9.7	13.2	5.52/6.79	−0.7	43.1
6d	39.5/42.6	7.3	9.9	6.14/6.72	+1.9	
Average		7.6	8.2		+0.4	
Standard deviation of mean		±0.5	±1.1		±0.3	26.4

of 8.2 ml. per 100 ml. ± 1.1 ml. to suggest that there is no significant change in red cell volume during the stasis induced by the cuff.

The mean venous oxygen saturation during the altitude experiments was 26.4 per cent. The arterial oxygen saturation was maintained at 55 to 65 per cent throughout the period of cuff application. The oxygen tension along the entire capillary was therefore much less than its normal value. On the other hand, in the subjects employed, repeated checks of systolic blood pressure during the course of the cuff application at altitude showed no significant change. The absence of a significant change in the mean fluid loss when using anoxia (7.9 ml. per 100 ml. at sea level and 6.3 ml. per 100 ml. at altitude) contrasts with the marked change found in the experiments with adrenalin. It suggests that the pressure in the capillaries and the rate of blood flow through the forearm were not significantly affected during these latter tests. The changes found in the percentage of protein in the filtrate can therefore be attributed to the reduction in oxygen tension of the blood in the vessels. The difference between the mean value of 1.2 ± 0.4 grams per cent obtained during the tests at altitude when contrasted with that of 0.4 ± 0.3 gram per cent for the controls, represents a definite increase in protein in the filtrate as a result of the change in venous oxygen saturation from the mean of 26.4 per cent at sea level to 11.4 per cent at altitude.

In Table V the evidence obtained from the 2 sets of experiments is summarized. When the data from the altitude work are considered in conjunction with the evidence obtained in the adrenalin studies a picture is obtained which suggests that anoxia has some effect upon capillary

TABLE IV

Fluid loss and per cent protein in filtrate with 60 mm. Hg congestion (arterial saturation 55 to 65 per cent)

Subject	Hematocrit	Fluid loss (hematocrit)	Fluid loss (hemoglobin)	Protein	Protein in filtrate	Saturation of venous blood
		ml. per 100 ml.		*grams*	*per cent*	
7j	47.2 49.4	5.6	5.6	6.83 7.41	+1.9	
7k	48.8 51.7	5.7	6.3	6.96 7.75	+0.7	
7l	44.2 48.6	9.2	6.4	6.79 8.06	+0.4	
7m	43.6 47.6	8.3	11.0	7.34 8.30	+1.8	
7n	43.8 46.8	6.5	8.1	6.21 6.72	+2.3	11.7
7o	45.7 48.7	6.0	3.2	6.04 6.52	+2.2	13.0
6e	41.4 44.5	7.0	3.9	6.41 7.48	−1.5	
6f	38.6 41.9	7.9	5.4	6.07 6.55	+2.8	
6g	42.8 47.5	9.6	9.7	5.80 6.89	+0.4	11.0
6h	42.7 47.4	9.9	8.2	6.04 7.10	+1.2	8.9
7p	44.9 45.8	2.0	0.6	7.00 7.13	+3.6	14.8
6i	41.5 42.5	2.4	0.8	5.48 5.76	−1.0	9.2
Average		6.7	5.8		+1.2	11.4
Standard deviation of mean		±0.7	±1.0		±0.4	

TABLE V

Summary of changes obtained in fluid loss and per cent protein in the filtrate when anoxia is added to a 60 mm. Hg congestion and adrenalin hypertension to an 80 mm. Hg congestion experiment

	Values based on changes in		Values based on changes in	
	Hematocrits	Hemoglobins	Hematocrits	Hemoglobins
	60 mm. Hg controls (23 expts.)		*60 mm. Hg with anoxia (12 expts.)*	
Mean fluid loss	7.6 ml. per 100 ml.	8.2 ml. per 100 ml.	6.7 ml. per 100 ml.	5.8 ml. per 100 ml.
Standard deviation	±0.5 ml.	±1.1 ml.	±0.7 ml.	±1.0 ml.
Mean protein in filtrate	+0.4 gram per cent		+1.2 gram per cent	
Standard deviation	±0.3 gram per cent		±0.4 gram per cent	
Mean venous O_2 saturation	26.4 per cent		11.4 per cent	
	80 mm. Hg control (9 expts.)		*80 mm. Hg with adrenalin (9 expts.)*	
Mean fluid loss	13.4 ml. per 100 ml.	12.8 ml. per 100 ml.	6.0 ml. per 100 ml.	6.6 ml. per 100 ml.
Standard deviation	±1.7 ml.	±1.8 ml.	±0.6 ml.	±0.8 ml.
Mean protein in filtrate	+0.8 gram per cent		−1.0 gram per cent	
Standard deviation	±0.5 gram per cent		±0.3 gram per cent	
Mean venous O_2 saturation	18.0 per cent		30.5 per cent	

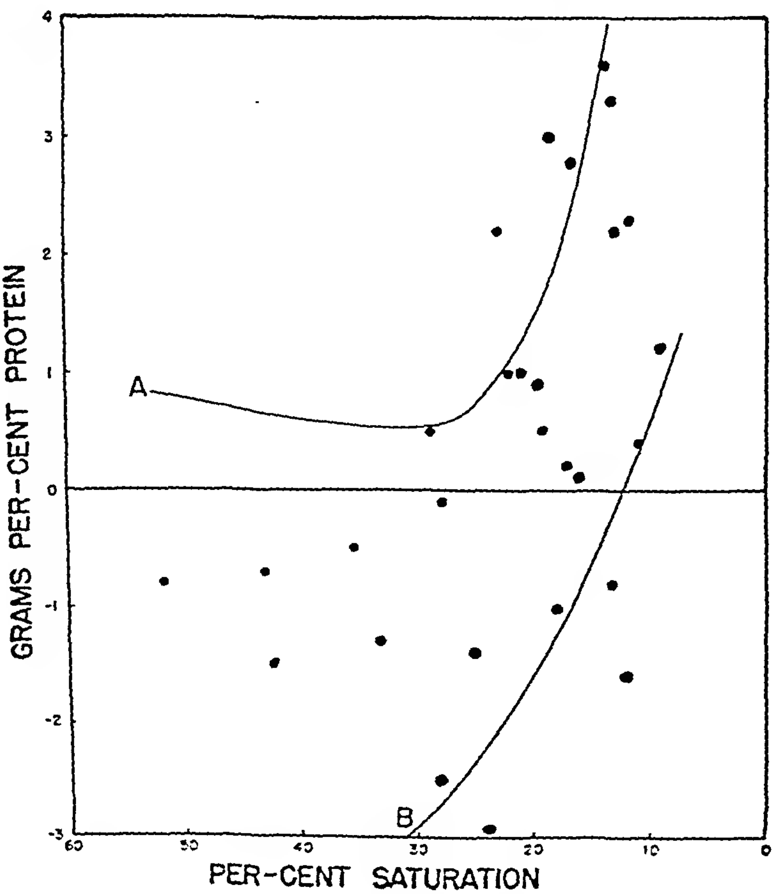

Fig. 2. Per Cent Protein in the Filtrate Plotted against Per Cent Oxygen Saturation of the Venous Blood

permeability in the forearm when the oxygen saturation falls below the general value of 25 per cent.[3]

In Figure 2 the values for protein in the filtrate are plotted against those for oxygen saturation in the venous blood. Inspection reveals a definite upward trend of these protein values at oxygen saturations of less than 25 per cent. Curve A, Figure 2, was obtained by adding to each determination of protein in the filtrate the positive deviations as calculated for Figure 1 and plotting the resulting points. Curve B was plotted in a similar manner using the negative deviations. They have no quantitative significance but represent an attempt to indicate qualitatively the skewing and relative accuracy of the data over the range of venous oxygen saturations considered. The convergence of these curves towards the higher values of protein in the filtrate is an expression of the lower random error at the higher values for protein and fluid loss. The marked negative bias of the error especially in the determinations with higher values of oxygen saturation probably accounts in large measure for those negative values observed . The essential feature portrayed is the increase in the protein in the filtrate with oxygen saturation values of less than 20 to 30 per cent. This is clearly shown on inspection of the data and can be confirmed by the above mathematical considerations.

DISCUSSION

The foregoing results suggest that it is necessary for the oxygen tension in the capillary bed of the human arm to drop to values below 15 to 25 mm. Hg corresponding to an oxygen saturation of 15 to 25 per cent, or an oxygen content of 4 to 6 vols. per cent in those with a normal hemoglobin content, before any significant changes in permeability to protein can be expected. However, if the oxygen tension falls below this value, there is good evidence that the anoxia is then of sufficient severity to increase capillary permeability even when it is of short duration. It is of interest that even during decompensation the oxygen content in blood from the antecubital vein of cardiac patients only rarely falls to such values (17). Nor are such values met in pneumonia except in moribund cases (18). In the cases of emphysema with cardiac failure described by Stead and Warren (8), in which there was no increase in protein in the edema fluid in spite of an arterial saturation of 50 to 60 per cent, the venous oxygen content was not recorded. It is possible that despite the severe anoxic anoxia the venous O_2 saturation was not below the critical level of 15 to 25 per cent. One reason for making this assumption is the fact that such patients often have polycythemia and their blood can therefore yield more oxygen before reaching any particular degree of desaturation than can the blood of those with a normal hemoglobin content.

In severe anemia the reverse of the above-mentioned condition occurs and subjects with hemoglobin concentration values of 20 per cent or less show very low venous oxygen saturation values of the order of 20 per cent (19). Strauss and Fox (20) have pointed out that the tendency to water retention on administration of sodium salts to those with severe anemia was not due to a low plasma protein level or to an increased venous pressure. Although endocrine factors (21) and the effect of changes in renal blood flow (22) must first be considered in assessing the cause of this water retention, it is possible that a part of the edema sometimes noted in severe anemia may be due to increased capillary permeability as a result of anemic anoxia. It is of interest in this connection to note the incidence of edema in cases of *erythroblastosis foetalis* occurring *in utero* (23). For in such cases in addition to the severe anemic anoxia present as a result of red cell destruction there is a considerable anoxic anoxia as a consequence of the incomplete oxygenation of the fetal blood by the placenta.

Aub and Cunningham (24) have shown that in irreversible traumatic shock the venous oxygen content may fall as low as 3 to 4 vols. per cent, representing saturations of the order of 15 to 20 per cent. It is possible that in this condition the venous oxygen tension may be low enough to

[3] Although venous oxygen determinations were not made for all of the 60 mm. cuff experiments (7 of 23 controls and 6 of 12 altitude experiments) the arterial oxygen saturation was reduced by the same amount in all cases. It is therefore assumed that the average venous oxygen saturation of those samples in which this measurement was not made would have been of the same order as the determined values.

result in a generalized increase in capillary permeability. This conclusion would fit with the observations of Fine and Seligman (25, 26). They have noted that following saline therapy in hemorrhagic shock, plasma proteins are carried out of the blood stream with the saline and that this occurs to a greater extent in irreversible than in reversible shock. These authors state their impression that "in irreversible shock the integrity of the capillaries as measured by the passage of radioactive protein may be impaired." Even in their more severe cases the venous oxygen saturation rarely fell as low as 5 vols. per cent and it would seem probable that only in the terminal phases of shock does the capillary oxygen content fall to values of 3 to 4 vols. per cent; that is, to levels where definite evidence of an increase in capillary permeability can be anticipated.

In their studies of the effects of anoxia on lymph production Maurer (5), working with the cervical lymph of dogs, and Warren and Drinker (6), with that from the lungs, both demonstrated a marked increase in lymph flow when the oxygen content in the inspired air was reduced to values of 10 per cent or less. Although they did not make direct estimations of venous oxygen tensions it is possible to interpolate figures from the data supplied by Davies (27) concerning the mean venous oxygen tension during varying degrees of anoxic anoxia. His data suggest that the oxygen saturation of the mixed venous blood in the lungs in their studies was probably not less than 35 per cent except in the cases where the oxygen percentage in the inspired air was considerably less than 10 per cent. Adrenalin secretion may be stimulated by anoxic anoxia of this degree and there is increased lymph production from the cervical lymphatics following the use of adrenalin (28). Although there was an increase in the total amount of lymph protein produced by their dogs, there was a decrease in the concentration of protein in the lymph collected during the anoxic episodes. This decrease is unexpected if the cause of the increased lymph flow is an increase in permeability of the capillary wall. If, however, the increased lymph production is due in large measure to changes in the blood flow through the capillary bed, then the fall in lymph protein concentration can be more readily explained. In those cases where the oxygen content in the inspired air was less than 10 per cent and in which the arterial oxygen saturation fell to less than 50 per cent, it is probable that the oxygen saturation of the mixed venous blood was within the critical range of 15 to 25 per cent necessary for the development of an increased capillary permeability. If our figures, determined from studies using the human forearm, also apply to the pulmonary capillary network, it is probable that in these cases anoxia played a direct part in the increased lymph flow.

Pochin (2) was led to his study of the relation of gross ischemia to edema formation by his observation of edema in a human limb, to which the circulation had been obstructed for 10 hours. It is possible to develop far higher grades of recoverable anoxia in isolated parts of the body such as a limb, ear, or finger, than in the entire organism which includes regions of very great sensitivity to anoxia such as the brain. Consequently, it is to be anticipated that evidence of edema formation as a result of anoxia of the capillary walls would be found in cases where the arterial blood supply to a region was grossly impaired. De Takats (29) has observed the frequent occurrence of edema in conditions such as traumatic angiospasm, acute peripheral trophoneurosis and even in Buerger's disease. He emphasizes that this edema will vanish after sympathectomy or sympathetic paralysis. These procedures do not necessarily relieve any venous obstruction that may be present in such cases. This points to a relief of the anoxia of the capillary wall, resulting from the improved blood flow, as the probable cause of the improvement of the edema. Homans (30) has recently confirmed this opinion that the edema so frequently observed in arterial spasm is due to anoxia of the capillary wall. Oschner and De Bakey (31) in a discussion of the mechanism of development of edema in thrombophlebitis, conclude that it is not solely due to the increase in venous pressure as a result of the obstruction to the venous drainage from the part. They consider that reflex vasospasm of the arteries is a most important factor and that as a result of the vasospasm there is a relative anoxia of the capillary endothelium with an en-

suing increase in permeability. If blocking the sympathetic pathways is effective in relieving arterial spasm, then the blood flow through the part increases, the oxygen saturation in the venous blood rises and at the same time the edema decreases. It would be of interest to know the oxygen saturation of blood draining from the edematous regions before and after sympathetic paralysis in such cases.

It is known that in the liver which largely depends on venous blood for nourishment, the oxygen tension in the blood in the hepatic veins may fall to very low levels. Thus Engel, Harrison, and Long (32) and also McMichael (33) note hepatic venous oxygen saturations in the cat of 3 to 30 per cent during hemorrhage. These low values were often obtained before the blood pressure had fallen significantly below its initial level. Frank, Seligman, and Fine (34) have associated the susceptibility of the liver to damage in shock with this peculiarity of the blood supply. It would be of interest to know what part anoxia of the liver capillaries might play in their known high permeability to protein (10) and whether the effects of anoxia on capillary permeability to protein differ in the different regions of the body. Finally, it may be noted that this work has been carried out with acute anoxia lasting for 30 minutes only. It is not known whether prolonging the duration of the anoxia would significantly raise the critical level of oxygen tension at which capillary damage first occurs. However, the failure of Stead and Warren (8) to observe an increase in protein in the extracellular fluid in their subjects with chronic anoxia would suggest that this does not occur.

The preceding observations indicate that anoxia commences to affect capillary permeability to the point of significant protein leakage at a very low oxygen tension. The result is that it usually requires a combination of one or more of the factors, ischemia, anoxic anoxia, anemia, or a great increase in tissue oxygen consumption, to attain a sufficiently low level. It may be concluded that in states of uncomplicated mild anoxic anoxia there is no reason to anticipate changes in the capillary permeability of the limbs. On the other hand, in conditions such as fatal asphyxia, irreversible shock, and ischemia in which the venous oxygen saturation may fall below the critical level of 15 to 25 per cent a significant increase in permeability probably occurs a few minutes after the commencement of such anoxia.

SUMMARY

1. A number of venous occlusion experiments of 30 minutes' duration were performed with and without anoxic anoxia and with and without adrenalin hypertension.

2. The calculated protein in the capillary filtrate was not increased until the local anoxia was of such a degree that the oxygen saturation in the venous blood draining from the arm was 15 to 25 per cent or less.

3. The experiments with adrenalin hypertension suggested that ischemic anoxia and not the concomitant increase in capillary pressure is responsible for the increased amount of protein found in the filtrate resulting from the use of an arm cuff at a pressure of 80 mm. Hg.

4. In states of mild anoxia there is no reason to anticipate changes in the capillary permeability of the limbs. However, in asphyxia, irreversible shock, and in severe local ischemia or other states in which the venous oxygen saturation may fall below a critical level of 15 to 25 per cent, a significant increase in permeability to protein probably occurs a few minutes after the commencement of such anoxia.

BIBLIOGRAPHY

1. Landis, E. M., Micro-injection studies of capillary permeability; effect of lack of oxygen on permeability of the capillary wall to fluid and to plasma protein. Am. J. Physiol., 1928, 83, 528.
2. Pochin, E. E., Edema following ischemia in the rabbit's ear. Clin. Sci., 1939–42, 4, 341.
3. Calvin, D. B., The effect of asphyxia on plasma volume and protein concentration. Am. J. Physiol., 1941, 133, 233.
4. Hopps, H. C., and Lewis, J. H., Studies on capillary permeability as affected by anoxemia. Am. J. Path., 1946, 22, 656.
5. Maurer, F. W., Effects of decreased blood O_2 and increased blood CO_2 on flow and composition of cervical and cardiac lymph. Am. J. Physiol., 1940, 131, 331.
6. Warren, M. F., and Drinker, C. K., The flow of lymph from the lungs of the dog. Am. J. Physiol., 1942, 136, 207.

7. McMichael, J., and Morris, K. M., Acute oxygen lack and capillary permeability in man. J. Physiol., 1936, 87, 74P.
8. Stead, E. A., and Warren, J. W., Protein content of the extracellular fluid in normal subjects after venous congestion and in patients with cardiac failure, anoxemia, and fever. J. Clin. Invest., 1944, 23, 283.
9. Henry, J. P., Klain, I., Movitt, E., and Meehan, J., On the effects of acute anoxia on the permeability of the capillaries in the human arm. CAM Report No. 497 to NRC Div. of Med. Sciences January 25, 1946. Summary Fed. Proc., 1946, 5, 44.
10. Landis, E. M., Capillary permeability and the factors affecting the composition of capillary filtrate. Ann. New York Acad. Sciences, 1946, 46, 713.
11. Landis, E. M., Jonas, L., Angevine, M., and Erb, W., The passage of fluid and protein through the human capillary wall during venous congestion. J. Clin. Invest., 1932, 11, 17.
12. Kunkel, P., Stead, E. A., and Weiss, S., Blood flow and vasomotor reactions in the hand, forearm, foot and calf in response to physical and chemical stimuli. J. Clin. Invest., 1939, 18, 225.
13. Allen, W. J., Barcroft, H., and Edholm, O. G., On the action of adrenalin on the blood vessels in human skeletal muscle. J. Physiol., 1946, 105, 255.
14. Chapin, M. A., and Ross, J. F., The determination of the true cell volume by dye dilution, by protein dilution and with radioactive iron: The error of the centrifuge hematocrit. Am. J. Physiol., 1942, 137, 447.
15. Henry, J., Greeley, P. O., Frykman, V. L., and Peterson, L., An indwelling needle for use in the radial artery. Science, 1946, 104, 299.
16. Griffith, F. R., Omachi, Akira, Lockwood, J. E., and Loomis, T. A., The effect of intravenous adrenalin on blood flow, sugar retention, lactate output and respiratory metabolism of peripheral (leg) tissues in the anesthetized cat. Am. J. Physiol., 1947, 149, 64.
17. Lundsgaard, C., Studies of oxygen in the venous blood. IV. Determinations on five patients with incompensated circulatory disturbances. J. Exper. Med., 1918, 27, 219.
18. Stadie, W. C., The oxygen of the arterial and venous blood in pneumonia and its relation to cyanosis. J. Exper. Med., 1919, 30, 215.
19. Sharpey-Schafer, E. P., Transfusion and the anemic heart. Lancet, 1945, 2, 296.
20. Strauss, M., and Fox, H. J., Anemia and water retention. Am. J. M. Sc., 1940, 200, 454.
21. Futcher, P., and Schroeder, H., Studies on congestive heart failure. II. Impaired renal excretion of sodium chloride. Am. J. M. Sc., 1942, 204, 52.
22. Merrill, A. J., Edema and decreased renal blood flow in patients with chronic congestive heart failure; evidence of "forward failure" as the primary cause of edema. J. Clin. Invest., 1946, 25, 389.
23. Diamond, L. K., Blackfan, K. D., and Baty, J., Erythroblastosis fetalis and its association with universal edema of the fetus, icterus gravis neonatorum and anemia of the newborn. J. Pediat., 1932, 1, 269.
24. Aub, J. C., and Cunningham, T. D., Studies in experimental traumatic shock. II. The oxygen content of the blood. Am. J. Physiol., 1920–21, 54, 408.
25. Fine, J., and Seligman, A. M., Traumatic shock. VII. A study of the problem of the "lost plasma" in hemorrhagic, tourniquet, and burn shock by the use of radioactive iodoplasma protein. J. Clin. Invest., 1944, 23, 720.
26. Fine, J., and Seligman, A. M., Traumatic shock. IV. A study of the problem of the "lost plasma" in hemorrhagic shock by the use of radioactive plasma protein. J. Clin. Invest., 1943, 22, 285.
27. Davies, B. D., The indirect measurement of mean venous oxygen tension during anoxia. J. Clin. Invest., 1944, 23, 666.
28. Drinker, C. K., Warren, M. F., Maurer, F. W., and and McCarrell, J. D. The flow, pressure and composition of cardiac lymph. Am. J. Physiol., 1940, 130, 43.
29. de Takats, G., Reflex dystrophy of the extremities. Arch. Surg., 1937, 34, 939.
30. Homans, J., Vasomotor and other reactions to injuries and venous thrombosis. Am. J. Med. Sc., 1943, 205, 313.
31. Ochsner, A., and DeBakey, M., Thrombophlebitis: The role of vasospasm in the production of the clinical manifestations. J. A. M. A., 1940, 114, 117.
32. Engel, F. L. Harrison, H. C., and Long, C. N. H., Biochemical studies on shock. 111. The role of the liver and the hepatic circulation in the metabolic changes during hemorrhagic shock in the rat and the cat. J. Exper. Med., 1944, 79, 9.
33. McMichael, J., The oxygen supply of the liver. Quart. J. Exper. Physiol., 1937, 27, 73.
34. Frank, H. A., Seligman, A. M., and Fine, J., Traumatic shock. XIII. The prevention of irreversibility in hemorrhagic shock by vivi-perfusion of the liver. J. Clin. Invest., 1946, 25, 22.

THE HEPATIC BLOOD FLOW AND SPLANCHNIC OXYGEN CONSUMPTION OF MAN—THEIR ESTIMATION FROM UREA PRODUCTION OR BROMSULPHALEIN EXCRETION DURING CATHETERIZATION OF THE HEPATIC VEINS[1]

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The hepatic blood flow was first estimated in normal man by Bradley, Ingelfinger, Bradley and Curry (1). Before applying this bromsulphalein procedure to clinical problems, it seemed advisable not only to confirm their measurements but to estimate hepatic blood flow by a fundamentally different method. The urea method for hepatic blood flow, worked out in the dog by Lipscomb and Crandall (2), has been adapted to normal unanesthetized human subjects. The hepatic venous-arterial oxygen difference, total oxygen consumption, and cardiac output have been measured in the same subjects. By multiplying the hepatic vein flow by the hepatic venous-arterial oxygen difference, one obtains an estimate of the splanchnic oxygen consumption.[2] The oxygen consumption and blood flow of the brain and kidney have been estimated by other investigators. Thus, the measurement of oxygen consumption and blood flow of the splanchnic area allows one to construct a fairly complete picture of the degree of oxygen utilization by and the blood flow through the most important areas of the body.

Urea, among the various metabolites involving the liver, has several attributes which adapt it as a test substance for the estimation of liver blood flow. As far as is known, urea is produced in the human organism only by the liver (3, 4) and, once formed, is not converted to other compounds. Urea is very diffusible not only through the various elements of the extracellular fluid but through cell membranes as well. Since the level of urea in arterial blood generally is constant over a period of several hours under conditions of fasting, urea must be lost from the body at the same rate as it is formed, and the total urinary excretion of urea under such conditions approximates hepatic production.

The hepatic blood flow can be calculated, by the "Fick principle," if one knows (*a*) the concentration of urea in the blood entering the liver (hepatic arterial and portal venous bloods), (*b*) the concentration of urea in the blood leaving the liver (hepatic venous blood), and (*c*) the quantitative excretion of urea in the urine during the period when the hepatic blood flow is being measured. Item (*c*) makes the urea method unadapted to the hepatic blood flow over a short period of time; the flow obtained is really the mean liver blood flow for the period of collection of the urine.

Samples of blood from the hepatic artery and portal vein cannot be obtained in man except by surgical means. Peripheral arterial blood has the same urea content as hepatic arterial blood. Bloods from the portal vein and femoral artery have been found to have identical urea contents in dogs (2), and it is reasonable to assume that this same circumstance holds for man. With this assumption, then, one can utilize the blood of the femoral or brachial artery in place of the true inflow blood of the liver for the determination of urea concentration. Blood from the hepatic vein of man can be obtained directly by the process of venous catheterization as described by Warren and Brannon (5).

METHODS

Patients, with the one exception noted, had all fasted 6 hours or longer prior to the test. The entire procedure

[1] This work was supported by a grant from the Life Insurance Medical Research Fund.

[2] The splanchnic area as used in this paper constitutes the liver plus the area drained by the portal vein. The splanchnic blood flow will be equal to the total hepatic blood flow, but the splanchnic oxygen consumption is greater than that of the liver because the oxygen consumption of all of the portal viscera is included.

was performed in an air conditioned room and excessive urea loss by perspiration was avoided. The urine sample was collected in the standing position by having the patient empty his bladder as completely as possible at the beginning and the end of the procedure. The time interval of collection of the urine averaged about 2 hours.

Catheterization of the hepatic veins

With the subject in the supine position on the fluoroscopic table, the catheter was inserted into a superficial arm vein under local procaine anesthesia. It was passed under fluoroscopic control into the superior vena cava, through the right atrium and thence into the hepatic veins (1, 5). A continuous infusion of 0.9 per cent sodium chloride solution was maintained through the catheter. The subject received on the average 200 to 300 ml. of the solution during the entire procedure. In most instances, the catheter entered one of the right hepatic veins. It was introduced so that its tip was located near the center of the right lobe of the liver as visualized by fluoroscopy in the frontal plane. If the catheter was placed farther out along the hepatic veins, it became more difficult to obtain blood samples because the single hole near the tip of the catheter would become occluded intermittently against the wall of the vein. In the larger radicles of the hepatic veins this difficulty is encountered uncommonly. The blood sample is withdrawn gently and necessarily slowly due to the small caliber of the catheter. Under these circumstances, and because of the rapid flow of blood through the hepatic veins, reflux of blood from the inferior vena cava, or right atrium in those cases in which the hepatic veins open directly into that chamber, would appear unlikely if it can occur at all.

Forty-milliliter samples of blood were collected in heparinized syringes from the hepatic vein and femoral artery either simultaneously or within 2 minutes of one another. After a 20- to 30-minute interval, second samples of both hepatic venous and arterial blood were withdrawn. In most instances, a blood sample was taken from the hepatic vein for measurement of oxygen content. The catheter was then partially withdrawn and placed either in the right atrium or preferably in the right ventricle or pulmonary artery. Arterial and mixed venous bloods were taken and a 2-minute sample of expired air collected in a Douglas bag to be used in determination of the cardiac output.

An attempt was made in each patient to assay the amount of anxiety produced in response to the entire procedure.

Determination of urea in blood

Because of the small difference in urea concentration between hepatic venous and femoral arterial blood, the determination of the levels of urea in the 2 bloods must be done with maximum accuracy. The method used was a modification of the urease method of Van Slyke and Cullen (6), which utilizes 20 ml. of blood in each assay, so that relatively large amounts of urea are being measured. Ten-milliliter samples were found to be too small.

Twenty milliliters of whole heparinized blood were pipetted into a 200-ml. volumetric flask and 100 mgm. of urease (Squibb double strength) in 2 ml. of distilled water were added. No additional buffer was used. After allowing the urease to act for 30 minutes, a tungstate filtrate was made from the blood-urease mixture. A 100-ml. aliquot of this filtrate was steam-distilled, using a macro-Kjeldahl distilling apparatus, after the addition of 100 ml. of saturated sodium tetraborate solution. The distillate was caught in an Erlenmeyer flask containing 20 ml. of 4 per cent boric acid solution and methyl red as an indicator, and titrated against 0.01 N sulphuric acid. All determinations were done in duplicate and the results were discarded unless they checked within 0.2 ml. of 0.01 N acid, which is equivalent to 0.06 mgm. of urea in the specimen, or .6 mgm. of urea per 100 ml. of whole blood. Most duplicate determinations checked within 0.1 ml. or less of 0.01 N acid. The accuracy of this method in detecting small differences in urea concentration in human blood was determined by means of recovery experiments. In each experiment, blood from a normal subject was divided into 4 portions of 20 ml. each. A known quantity of urea in aqueous solution, usually 1.0 mgm. per 100 ml. of blood, was added to 2 samples, and an equal amount of distilled water to the other pair. All 4 were then analyzed for urea content by the procedure outlined above. No experiment was considered satisfactory if the variation between the duplicate determinations exceeded 0.2 ml. of 0.01 N acid. The recoveries in 14 such experiments ranged from 79 to 130 per cent with a mean recovery of 102 ± 12.4 per cent. At an urea difference of 1 mgm. per 100 ml., this equals 0.12 mgm. Twice this standard deviation is 0.25 mgm. per 100 ml. This result would indicate, in the comparison of 2 values (such as an arterial and an hepatic venous urea level) each of which may have a deviation of ± 0.25 mgm. per 100 ml., that a difference of less than 0.5 mgm. per 100 ml. cannot be considered significant. The accuracy of the method was further investigated by performing 8 analyses on a single specimen of blood. The values obtained for blood urea concentration, in mgm. per 100 ml., were: 14.2; 13.8; 13.9; 13.9; 13.9; 14.2; 13.9; 14.2. The mean of these results is 14.0 ± 0.17 mgm. per 100 ml.

Determination of urea in urine

A volume of urine approximately equal to 1 minute's output was diluted with 20 ml. of distilled water and the ammonia removed with 3 grams of permutit. A 10-ml. aliquot of this ammonia-free filtrate was treated for 30 minutes with 1 ml. of a 5 per cent solution of urease (Squibb, double strength). A tungstate filtrate was then prepared and a large aliquot, usually 50 ml., was steam-distilled with sodium tetraborate. The procedure from this point on is the same as for blood.

The oxygen content of the mixed venous and arterial bloods was determined by the method of Van Slyke and Neill (7). The oxygen and carbon dioxide contents of the expired air were measured by the method of Haldane.

Experimental procedure for bromsulphalein method

The general conditions of study and the procedure of catheterization were the same as for the urea method. Bromsulphalein in physiological saline was administered as described by Bradley *et al*, using a tunnel clamp (8) and a calibrated Murphy drip. A priming dose of 150 mgm. of bromsulphalein was used. Then an equilibration period of about 30 minutes, with the constant infusion running, is allowed before blood samples are withdrawn. These were then taken simultaneously from the femoral artery and hepatic vein at intervals of approximately 10 minutes.

The bromsulphalein in the serum was determined by means of the Beckman spectrophotometer at a wave length of 580 mμ. As a rule, 1 ml. of serum was diluted with 5 ml. of 0.9 per cent sodium chloride solution and 0.1 ml. of 10 per cent sodium hydroxide added just before reading. All determinations were made in duplicate with identical values usually being obtained.

Plasma volume determinations were done on every patient whose hepatic blood flow was estimated by the bromsulphalein method. The 10-minute Evans blue (T-1824) technique was used. The plasma volume was utilized in calculating the hepatic blood flow in the presence of a rising or falling arterial serum concentration of bromsulphalein (1). No data are included if the bromsulphalein concentration was changing at a rate exceeding 0.0002 mgm. per ml. of serum per minute.

RESULTS

Hepatic blood flow by the urea method

Fairly complete sets of data were collected on 11 males. Six of these had no disease which is known to involve the liver (epilepsy, low back strain, psychoneurosis, herniated intervertebral disc, brachial neuritis, lacerated thumb). Five were fasting for 6 hours or longer; the sixth was studied 2 hours after his usual lunch. The 5 other patients were well convalescent from acute infection (acute bronchitis, gonococcal arthritis, pneu-

TABLE I

Hepatic blood flow by the urea method—observations on 6 normal and 5 convalescent subjects

Subject	Age	Surface area	Hepatic venous-arterial urea difference	Urinary urea excretion	Urine volume	Hepatic blood flow	Oxygen consumption	Metabolic rate—deviation from normal basal	Arterial oxygen content	Arterial-mixed venous oxygen difference	Arterial-hepatic venous oxygen difference	Cardiac index	Liver blood flow as percentage of cardiac output	Splanchnic oxygen consumption	Splanchnic oxygen consumption as percentage of total oxygen consumption	Estimated anxiety
	yrs.	*sq. meters*	*mgm. per 100 ml.*	*mgm. per min.*	*ml. per min.*	*liters per min. per sq. m.*	*ml. per min. per sq. m.*	*per cent*	*volumes per cent*			*liters per min. per sq. m.*	*per cent*	*ml. per min. per sq. m.*	*per cent*	*0 = none to 4+ = marked*
5 Normal, fasting subjects																
J. M.	27	1.70	0.72	12.7	4.4	1.0	174	+27	16.8	4.8	4.4	3.6	29	45	26	±
R. L.	36	1.89	1.02	9.8	2.7	0.5	155	+23	19.0	3.2	4.1	4.8	11	21	14	±
C. S.	34	1.74	0.93	15.2	1.5	0.9			18.3	4.1	4.9			46		0
H. B.	25	1.75	0.93	10.1	3.4	0.6	152	+26	19.3	3.6	5.5	4.9	13	34	22	2+
S. W.	56	1.78	0.65	13.8	2.0	1.2										0
5 Fasting subjects, convalescing from acute infections																
M. H.	16	1.75	0.51	13.3	5.0	1.5	216	+41	18.7	5.1	4.1	4.2	35	61	28	4+
E. A.	21	1.78	1.14	15.3	0.5	0.8	147	+4	15.8	3.9	3.5	3.8	20	27	18	±
H. F.	18	1.87	0.72	13.7	0.9	1.0	160	+8	15.9	3.8	5.8	4.2	24	59	37	3+
G. T.	22	1.85	0.81	15.0	4.9	1.0										2+
C. N.	33	1.70	0.55	14.9	1.5	1.6			18.7							4+
Average values						1.0	167	+22	17.8	4.1	4.6	4.2	22	42	24	
1 Normal subject, non-fasting																
W. B.	33	1.92	1.08	24.3	2.3	1.2	171	+26	17.2	3.4	5.6	5.0	23	53	31	1+

mococcal pneumonia—2, and mild typhus fever). The data on both groups of patients are recorded in Table I. There appeared to be no significant differences in the findings on the 5 normal, fasting subjects as compared with the 5 who were convalescent from acute infections. Accordingly, both groups are combined in summarizing results.

The levels of urea in the arterial blood fell between 16.4 and 38.3 mgm. per 100 ml., and the levels in the hepatic venous blood between 17.3 and 39.4 mgm. per 100 ml. Hepatic venous-femoral arterial urea differences ranged from 0.51 to 1.08 mgm. per 100 ml. of blood. The rate of excretion of urea in the urine varied between 9.8 and 15.3 mgm. per minute, for an average of 13.4 mgm. per minute. This has been determined on an additional 8 subjects on whom liver blood flows were not measured. In these individuals, the urinary excretion of urea lay between 12.3 and 20.4 mgm. per minute with an average of 16.2 mgm. per minute. There was no correlation of urinary excretion of urea with the minute volume of urine in the range encountered (0.5 to 5.0 ml. per minute). The 1 subject (W. B.) who was studied 2 hours after a meal demonstrated a rate of urea excretion of 24.3 mgm. per minute.

The total hepatic blood flows for the 10 fasting patients range from 1.0 to 2.7 liters per minute. When calculated per square meter of body surface, the range of flows is 0.5 to 1.6 liters per minute with an average flow of 1.0 liter per minute. The hepatic blood flows comprise from 11 to 35 per cent of the total cardiac output, averaging 22 per cent.

Total oxygen consumptions were measured in 6 patients and ranged from 147 to 216 ml. per minute per square meter of body surface. These values are in general higher than normal basal oxygen consumption, which is not unexpected in view of the fact that the oxygen consumption was determined near the end of a somewhat trying 2-hour procedure. Every effort was made, however, throughout each study to allay anxiety and keep the subject as comfortable as possible.

The arterial-mixed venous oxygen differences ranged from 3.2 to 5.1 volumes per cent, and the arterial-hepatic venous oxygen differences from 3.5 to 5.8 volumes per cent. In 3 instances the arterial-mixed venous oxygen difference exceeded the arterial-hepatic venous difference, and in 4 subjects the situation was the reverse. It must be remembered that the oxygen contents of mixed venous blood and hepatic venous blood were never determined simultaneously. There was in general a 5- to 10-minute delay involved in moving the catheter from the hepatic vein into the right heart and collecting the mixed venous blood specimen. The cardiac indices (outputs per minute per square meter) varied from 3.6 to 4.9. The average value for cardiac index was 4.2 liters per minute per square meter, which is about 30 per cent above normal but which is in keeping with the degree of anxiety which was generally encountered. The splanchnic oxygen consumption varied from 21 to 61 ml. per minute per square meter in the 7 fasting subjects in whom it was measured. These figures average 42 ml. per square meter and equal from 14 to 28 per cent of the total oxygen consumption.

The data for hepatic blood flow, cardiac output, splanchnic oxygen consumption, and total oxygen consumption for the 1 non-fasting subject studied do not vary significantly from the corresponding data for the fasting subjects.

Hepatic blood flow by the bromsulphalein method

The data for the 9 fasting subjects whose hepatic blood flows were estimated by the bromsulphalein method are recorded in Table II. One female subject (J. E.) is included in this group. Six subjects had conditions thought not to involve the liver (brachial neuritis, psychoneurosis—2, latent bronchial asthma, no disease, and asymptomatic neuro-syphilis). The other 3 subjects were well convalescent from mild, acute infections (pneumococcal pneumonia—2, primary atypical pneumonia—1). Since there is no apparent difference in the results on the normal subjects as compared with the convalescents, the 2 groups of data may be combined.

The range of total hepatic blood flows was from 0.6 to 1.2 liters per minute per square meter, averaging 0.8 liter. This represents from 14 to 26 per cent of the cardiac output, or 19 per cent as an average. Two subjects (H. B. and H. F.) had liver blood flow estimated simultaneously by the urea and bromsulphalein methods with values for H. B. of 0.6 liter per minute per square meter by

TABLE II

Hepatic blood flow by the bromsulphalein method—observations on 6 normal and 3 convalescent subjects

Subject	Age	Surface area	BSP concentration of arterial serum	Hepatic extraction of BSP	Total removal rate of BSP	Hepatic blood flow	Oxygen consumption	Metabolic rate—deviation from normal basal	Arterial oxygen content	Arterial-mixed venous oxygen difference	Arterial-hepatic venous oxygen difference	Cardiac index	Liver blood flow as percentage of cardiac output	Splanchnic oxygen consumption	Splanchnic oxygen consumption as percentage of total oxygen consumption	Estimated anxiety
	yrs.	*sq. meters*	*mgm. per 100 ml.*	*per cent*	*mgm. per min.*	*liters per min. per sq. m.*	*ml. per min. per sq. m.*	*per cent*	*volumes per cent*			*liters per min. per sq. m.*	*per cent*	*ml. per min. per sq. m.*	*per cent*	*0 = none to +4 = marked*
6 Normal, fasting subjects																
H. B.	25	1.75	1.23	54	4.76	0.7	152	+26	19.3	3.6	5.5	4.9	14	40	26	+3
W. J. B.	31	1.97	0.88	31	6.26	1.0	138	+3	19.4	3.7	5.1	3.8	26	56	41	+2
L. M.	24	1.85	2.66	47	7.48	0.6	156	+7	18.4	3.8	4.3	4.1	15	26	17	+1
M. A.	23	1.68	1.75	44	7.34	0.9	178	+28	17.6	4.5	4.0	4.0	22	37	21	+2
E. F.	34	1.87	2.58	25	7.83	1.0	172	+26	16.6	3.8	4.9	4.5	22	49	28	+3
J. W.	47	1.57	1.59	52	5.16	0.7										0
3 Fasting subjects convalescing from acute infections																
H. F.	18	1.87	2.34	35	7.60	0.8	160	+8	15.9	3.8	5.8	4.2	19	44	28	+3
J. E.	26	1.52	2.87	44	7.27	0.6	130	+4	14.8	3.3	4.7	4.0	15	28	22	+2
H. H.	16	1.62	1.40	29	4.83	1.2										+3
Average values						0.8	155	+15	17.4	3.8	4.9	4.2	19	40	26	

urea and 0.7 by BSP, and for H. F. of 1.0 by urea and 0.8 by BSP.

Oxygen consumption, which was measured in 7 of the 9 subjects, ranged from 130 to 178 ml. per minute per square meter. The corresponding metabolic rates vary from plus 3 to plus 28 per cent of the normal basal metabolic rate, values again in keeping with the amount of anxiety expected and observed. The arterial-mixed venous oxygen differences in 7 subjects ranged from 3.3 to 4.5 volumes per 100 ml. of blood, and the arterial-hepatic venous oxygen difference from 4.0 to 5.8 volumes per cent. In 6 of the 7 cases the arterial-hepatic venous difference exceeded the arterial-mixed venous difference. The cardiac indices varied from 3.8 to 4.9 liters per minute per square meter, with an average of 4.2 liters. The splanchnic oxygen consumption was determined in 7 subjects and varied from 26 to 56 ml. per minute per square meter with an average of 40 ml. These consumptions represent from 17 to 41 per cent of the total oxygen consumption.

In addition to the 11 subjects whose data for hepatic blood flow by the urea method are included in Table I, the urea procedure was carried out in 3 other individuals. The first of these (W. J. B.) had an hepatic venous-arterial urea difference of only 0.4 mgm. per 100 ml. of blood. This cannot be considered significant in light of the degree of accuracy of the method. A second patient (W. S.) showed the concentration of urea in the femoral arterial blood to be higher, by 0.8 mgm. per 100 ml., than the concentration in the hepatic venous blood. Fluoroscopically, the catheter appeared to be in the hepatic veins but the observer could have been mistaken. The third subject (H. H.) also demonstrated lower levels of urea in the hepatic venous blood than in arterial blood. The difference between the first arterial and hepatic venous samples was 0.4 mgm. per 100 ml.—not a significant difference. The second arterial and hepatic venous samples showed a urea difference of 1.05 mgm. per 100 ml. This hepatic venous sample was obtained with considerable difficulty due to

partial occlusion of the catheter by blood clot. A few minutes later, the catheter was completely thrombosed. This situation may have interfered with the clearing of the catheter of saline before the blood specimen was taken and thus resulted in dilution of the sample with the residual saline in the line. These 3 subjects had urinary urea excretions of 13.9, 12.3, and 19.3 mgm. per minute, respectively. Two of these subjects (W. J. B. and H. H.) had simultaneous determinations of liver blood flow by bromsulphalein with values of 1.0 and 1.2 liters per minute per square meter.

Two of the subjects (H. B. and C. S.) who are included in Table I showed, over time periods of 34 and 10 minutes, respectively, corresponding falls in arterial and hepatic venous urea concentrations with maintenance of essentially constant hepatic venous-arterial urea differences. In H. B. the arterial urea level fell from 16.4 to 15.6 mgm. per 100 ml. while the hepatic venous level changed from 17.3 to 16.5 mgm. per 100 ml. Thus, the hepatic venous-arterial urea difference remained constant at 0.9 mgm. per 100 ml. C. S. showed a fall in arterial urea concentration from 27.6 to 27.1 mgm. per 100 ml.; at the same time the hepatic venous concentration fell from 28.5 to 27.8 mgm. per 100 ml. The corresponding urea differences are 0.9 and 0.7 mgm. per 100 ml. It would not seem likely that the corresponding falls in arterial and hepatic venous urea concentrations were due to temporary dilution of the blood volume by the saline infusion through the catheter. In all other subjects the 2 arterial blood urea levels were identical, within the limits of error of the method.

DISCUSSION

The estimation of hepatic blood flow by the urea method has several disadvantages. The hepatic venous-arterial urea difference is small and requires a meticulous technique for its determination. Even so, small technical errors greatly influence the calculated hepatic blood flow. Because urea is so diffusible throughout the body fluids, *i.e.*, throughout a volume of water equal to approximately ⅔ of the body weight, the retention of a considerable proportion of the urea produced by the liver over a short period of time could occur without a significant rise in urea concentration in the arterial blood. Under such conditions, the urinary excretion of urea would not quantitatively represent urea production by the liver. Hepatic production and renal excretion of urea are probably at or close to equilibrium, however, under the resting and fasting conditions of this study. Kidney function may be affected by such factors as pain and anxiety, which were present in small to moderate degree during the test. The collection of the urine, for determination of urea output, over a period of 2 hours or longer would seem to reduce any effect of pain or anxiety on urea excretion.

The bromsulphalein method gives reproducible results as our average value for hepatic blood flow, 0.8 liter per minute per square meter, agrees closely with that of Bradley *et al* of 0.9 liter (1). The bromsulphalein method, however, involves several assumptions: (*a*) that bromsulphalein is removed from the blood stream only by the liver, (*b*) that the concentration of bromsulphalein in the portal vein is the same as that in peripheral systemic arterial or venous blood, (*c*) that the percentage of dye extracted by the portion of the liver which is catheterized represents the extraction percentage for the liver as a whole, and (*d*) that bromsulphalein and/or catheterization of the hepatic veins do not in themselves alter liver blood flow. Those assumptions which are inherent to catheterization of an hepatic vein are involved in the urea method as well.

One must conclude that the urea method is not only technically more difficult but probably considerably less accurate in estimating hepatic blood flow than is the bromsulphalein method. The urea procedure has served a useful purpose in demonstrating that the fundamental assumptions utilized in the dye method are probably correct because similar values for hepatic blood flow can be obtained by an entirely different method. We now feel safer in applying the bromsulphalein method to physiological and clinical problems.

If the values for hepatic blood flow and splanchnic oxygen consumption by both methods are combined, the average hepatic blood flow, 0.9 liter per minute per square meter, is 20 per cent of the cardiac output, and the average splanchnic oxygen consumption of 41 ml. per minute per square meter equals 25 per cent of the total oxygen con-

TABLE III

Comparison of approximate values for blood flow, arterio-venous oxygen difference, and oxygen consumption of brain, kidney and liver of man

	Visceral blood flow		Arterio-venous oxygen difference		Visceral oxygen consumption		Visceral blood flow as percentage of cardiac output		Visceral oxygen consumption as percentage of total oxygen consumption	
	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.
	ml. per 100 grams of tissue per minute		*vol. per cent*		*ml. per 100 grams of tissue per minute*					
Brain	59–75	66	5.7–7.2	6.5	3.9–4.9	4.4	18–21	20	20–30	24
Kidney	240–510	330	1.9–2.5	2.2	4.1–9.9	8.2		20		12
Liver (plus viscera drained by portal vein)										
(*a*) Urea method	58–185	117	3.5–5.8	4.6	2.4–7.0	5.3	11–35	22	14–37	24
(*b*) BSP method	69–138	92	4.0–5.8	4.9	3.0–6.5	4.6	14–26	19	17–41	25

The average body size is assumed to be 1.73 square meters and average visceral weights to be: brain, 1400 grams; kidneys, 170 grams each; and liver, 1500 grams. Data for the brain are from Kety and Schmidt (10), and for the kidney from Warren *et al* (11), Chasis *et al* (12), and Bradley and Halperin (13).

sumption. Both the hepatic blood flow and the splanchnic oxygen consumption vary over a wide range from person to person. These variations are not appreciably decreased by relating the values to the surface area of the subject. The splanchnic oxygen consumption was not closely correlated with the total oxygen consumption and only poorly with the hepatic venous-arterial oxygen difference. Both the total oxygen consumption and the cardiac index were somewhat higher than normal in a number of these subjects due, it is thought, to anxiety. It remains to be determined whether more consistent figures can be obtained by studying larger groups of more basal subjects or by studying all subjects after a procedure which will increase the work of the liver and tend to nullify the effects of anxiety. In the study of the cardiac output in the average patient the second technique has been the more useful; the spread in the resting values is considerably reduced by light exercise (9).

The liver is compared, in Table III, with the brain and kidney as to approximate blood flow, arterio-venous oxygen difference and oxygen consumption. The oxygen values recorded for the liver, of course, do not represent oxygen absorbed and utilized only by that viscus, but oxygen metabolized by all the viscera drained by the portal vein as well. The liver and its associated viscera lie between the brain and the kidneys in respect to blood flow per 100 grams of tissue and percentage of oxygen extracted from the arterial blood. The brain has a relatively low blood flow and higher arterio-venous oxygen difference; the kidneys have a very high blood flow but a low A-V difference. The liver and brain are about equal in oxygen consumption per 100 grams of tissue; the kidneys have a considerably higher consumption. Each viscus receives around 20 per cent of the resting cardiac output. Together, these viscera account for about 60 per cent of both the cardiac output and the total oxygen consumption of the body at rest.

SUMMARY AND CONCLUSIONS

1. The hepatic blood flow has been estimated utilizing the Fick principle with urea as the test substance.

2. The hepatic blood flow in 10 normal or convalescent subjects ranged from 0.5 to 1.6 liters per minute per square meter with a mean of 1.0 liter. Hepatic blood flow was estimated on 9 similar subjects by the bromsulphalein method with a range of flows from 0.6 to 1.2 liters per minute per square meter and an average of 0.8 liter. These hepatic blood flows by the 2 methods comprise 22 per cent and 19 per cent, respectively, of the average cardiac outputs.

3. Arterial-hepatic venous oxygen differences, determined at the same time as the hepatic blood flows, provided estimation of the splanchnic oxygen consumption. By the urea method, this ranged from 21 to 61 ml. of oxygen consumed per

minute per square meter with an average of 42 ml. By the bromsulphalein method, the splanchnic oxygen consumption varied between 26 and 56 ml. per minute per square meter and averaged 40 ml. These averages make up 24 per cent (for the urea method) and 26 per cent (for the bromsulphalein method) of the total oxygen consumption.

4. Because of the small difference in urea concentration between arterial blood and hepatic venous blood, a meticulous technique is required for the urea method, and small technical errors make large variations in the calculated hepatic blood flow. These disadvantages render the urea procedure less suitable for clinical use than is the bromsulphalein method. The fact that these 2 widely different methods give comparable estimates of hepatic blood flow would indicate that the basic assumptions underlying the bromsulphalein method are valid.

ACKNOWLEDGMENT

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BIBLIOGRAPHY

1. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., Estimation of hepatic blood flow in man. J. Clin. Invest., 1945, 24, 890.
2. Lipscomb, A., and Crandall, L. A., Hepatic blood flow and glucose output in normal unanesthetized dogs. Am. J. Physiol., 1947, 148, 302.
3. Bollman, J. L., Mann, F. C., and Magath, T. B., Studies on the physiology of liver; effect of total removal of the liver on formation of urea. Am. J. Physiol., 1924, 69, 371.
4. Maddock, S., and Svedberg, A., The effect of the total removal of the liver of the monkey. Am. J. Physiol., 1938, 121, 203.
5. Warren, J. V., and Brannon, E. S., A method of obtaining blood samples directly from the hepatic vein in man. Proc. Soc. Exper. Biol. and Med., 1944, 55, 144.
6. Van Slyke, D. D., and Cullen, G. E., The determination of urea by the urease method. J. Biol. Chem., 1916, 24, 117.
7. Van Slyke, D. D., and Neill, J. M., The determination of gases in blood and other solutions by vacuum extraction and manometric measurement. J. Biol. Chem., 1924, 61, 523.
8. Bradley, S. E., A tunnel clamp for use in controlling infusion rates. Science, 1947, 105, 214.
9. Hickam, J. B., and Cargill, W. H., Unpublished observations.
10. Kety, S. S., and Schmidt, C. F., The effects of active and passive hyperventilation on cerebral blood flow, cerebral oxygen consumption, cardiac output, and blood pressure of normal young men. J. Clin. Invest., 1946, 25, 107.
11. Warren, J. V., Brannon, E. S., and Merrill, A. J., A method of obtaining renal venous blood in unanesthetized persons with observations on the extraction of oxygen and sodium para-aminohippurate. Science, 1944, 100, 108.
12. Chasis, H., Redish, J., Goldring, W., Ranges, H. A., and Smith, H. W., The use of sodium para-aminohippurate for the functional evaluation of the human kidney. J. Clin. Invest., 1945, 24, 583.
13. Bradley, S. E., and Halperin, M. E., Renal oxygen consumption and sodium para-aminohippurate extration in normal man during abdominal compression. Federation Proc., 1947, 6, 80.

THE URINARY EXCRETION OF RADIOIODINE IN VARIOUS THYROID STATES

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Radioiodine and natural iodine are chemically and physiologically identical, providing the former is present in quantities so small as to be without biologic effects due to radiation *per se*. A minute quantity of radioiodine may be used to trace, by its radioactivity, the course of a given quantity of iodine through various chemical and biologic reactions. Hertz (1, 2) was the first to call attention to the unique possibilities of this method in the study of thyroid function. Hamilton and Soley (12) were the first to apply it to the clinical investigation of the human thyroid. Radioiodine tracers have subsequently been utilized in many studies dealing with the thyroid of laboratory animals, *in vitro* experiments and clinical investigations. The method has been applied to the study of the biosynthesis of thyroid hormone (1, 3 to 9), the action of thyrotrophic hormones (4, 10), the action of goitrogens (5, 11) and of other factors influencing the function of the thyroid (6), the functional activity of various thyroid tumors and a number of other problems.

It is possible that radioiodine may prove most useful in the study of thyroid function in man. To date, relatively few definitive clinical studies employing this technique have been published. The first was that of Hamilton and Soley (12), who compared the collection by the thyroid of iodine labeled with radioiodine and its excretion in the urine and feces in normal subjects and in patients having various thyroid disorders. Radioiodine in the thyroid was measured directly by the recording of its gamma radiation by means of a Geiger-Müller counter placed over the trachea and in some instances by analysis of surgically removed specimens of thyroid tissue. It was found that an orally administered dose of labeled iodine was absorbed very rapidly and could be detected in the thyroid within 20 minutes. Normal subjects excreted 74 to 89 per cent of the dose in the urine during a period of 5 days, 53 to 81 per cent appearing during the first 24 hours. Two myxedematous patients excreted 91 and 94 per cent, respectively, in the urine during 5 days but at a slower rate than did the normals. Patients who had hyperthyroidism excreted as much in the urine as did the normals. This finding may have been due to the fact that most of the patients had received strong solution of iodine (Lugol's solution) and in part to the very large amount of iodide (14 mgm.) which was administered as a labeled dose. Fecal excretion was variable but averaged only about 1 per cent of the dose.

In another series of *in vivo* measurements employing the same dose of iodide, Hamilton and Soley (13) recorded characteristic collection curves for various thyroid states. The curve typical of normal thyroids was a smooth curve which leveled off to a flat plateau in 2 days. Iodine collection by thyroids of hyperthyroid patients was greater and much more rapid than in normal thyroids. It rose to a peak within 4 to 8 hours and thereafter decreased almost as rapidly to a plateau lower than that of the normal thyroids. This curious phenomenon was also observed in 2 children who had goiters and hypothyroidism. When a dose of 0.1 microgram of sodium iodide was given instead of 14 mgm. such rapid loss of radioiodine from the thyroid was not observed (14).

Hertz, Roberts and Salter (3) studied 22 patients who had exophthalmic goiter and compared these with 2 normal subjects. The quantity of radioactivity was measured in the urine; that in the thyroid was measured *in situ* by applying a Geiger-Müller counter to the neck. Thyroid tissue was obtained in some cases following thyroidectomy. This was fractionated into diiodotyrosine-like and thyroxine-like fractions, which were also analyzed for radioactivity. The collection by thyroids in untreated exophthalmic goiter averaged

80 per cent if the accompanying dose of sodium iodide was small; when the latter exceeded 2 mgm. the uptake of radioiodine was smaller. Previous treatment with iodine reduced the quantity collected. The proportion of radioiodine which appeared in the thyroxine-like faction of thyroid tissue varied directly with the time intervening between the administration of the tracer and thyroidectomy.

Rawson and his associates (15, 16) studied the urinary excretion of radioiodine in a series of patients with various thyroid states. A patient who had a very large hyperplastic goiter resulting from treatment with potassium thiocyanate excreted only 24 per cent of a tracer dose of radioiodine, whereas a patient who had a similar goiter induced by administration of thiouracil excreted all of the tracer dose. The effect of thiouracil was also studied in a series of patients who had exophthalmic goiter and who were prepared for operation with this drug. The major part of the radioiodine appeared in the urine and very little was found to be in the thyroid. In 5 normal subjects, a total of 80 per cent of the dose appeared in the urine. A series of untreated thyrotoxic patients excreted an average of 16 per cent of the dose in the urine.

Leiter, Seidlin, Marinelli and Baumann (8) have studied the behavior of radioiodine tracers in 2 patients who had hyperfunctioning and metastatic thyroid adenocarcinomas. Both subjects excreted radioiodine in a manner comparable to that observed in cases of exophthalmic goiter. In one case thiouracil effectively reduced collection by the thyroid and in the other, in which the thyroid gland had previously been totally ablated, it proved equally effective in preventing collection of radioiodine by the metastatic thyroid tissue (17).

Leblond, Fertman, Puppel and Curtis (7) studied the form in which iodine is stored in the thyroid in cases of goiter. They found that less than 10 per cent of the quantity collected by the thyroid remained in the form of iodide; the rest was converted to diiodotyrosine or thyroxine. Comparable observations had been made earlier by Hamilton and Soley and their associates (14) in 2 cases of goiter with hypothyroidism.

All of the clinical reports which have appeared have dealt with the proportion of a given dose collected by the thyroid or excreted in the urine, or with the chemical form in which it was stored or secreted by the thyroid. No attempt has been made, thus far, to investigate the rates at which collection, excretion or secretion of iodine occurs. The collection curves reported by Hamilton and Soley are an exception in that the characteristic forms of these curves imply important differences in the dynamics involved although the differences noted were not expressed quantitatively.

The present study was undertaken to investigate the dynamics of iodine metabolism by the tracer method, and to determine, if possible, the rate at which radioiodine is collected by the thyroid, disappears from the blood and is excreted in the urine. The study was undertaken in the hope that this approach might provide the tracer method with greater precision when applied to various aspects of thyroid function in man.

The data to be presented concern chiefly the urinary excretion of radioiodine. Subsequent studies will deal with the concentration of radioiodine in blood and its collection and secretion by the thyroid as measured *in vivo*. Urinary measurements possess the disadvantage of not being directly concerned with thyroid function. There are, however, 2 reasons for directing attention first to urinary measurements: 1. The urine is the principal route for the disposal of an ingested dose of iodine in euthyroid or hypothyroid subjects. 2. The analysis of radioiodine in urine is technically much simpler and more accurate than its measurement in blood samples and much more accurate than present methods for measuring radioactivity *in vivo*.

METHODS

Iodine of mass 131 having a half life of 8.0 days was obtained from the Clinton Laboratories, where it had been prepared by the bombardment of metallic tellurium with slow neutrons in the chain-reacting pile. The isotope was supplied in solution as carrier-free iodide, with minute amounts of tellurium, sulfates and oxalates as contaminants. The solution was adjusted to pH 8.0, calcium chloride was added to precipitate oxalate, sodium iodide solution was added as carrier and the volume was adjusted so that 1.0 cc. contained 5.0 micrograms of sodium iodide and represented 500 microcuries as calculated from the stated activity of the sample as received. The usual tracer dose was 100 microcuries of I^{131} with a

total of 100 micrograms of sodium iodide as carrier.[1] In the earlier studies, the tracer doses were given in the morning after breakfast. Later they were given while the subject was fasting because it was found that food appeared to delay absorption appreciably.

Tracers usually were given to patients as outpatients. Instructions regarding the exact collection of specimens of urine were given with great care. They were first reviewed with the patient by the physician, repeated by the technician at the time the tracer was given and emphasized by means of a printed sheet on which the procedure for collection of urine was explicitly prescribed. If after diligent questioning at the time when specimens were returned, an error in collection of specimens was discovered, the data were discarded. In most cases the urine was collected every 6 hours for the first 24 hours and in 24-hour samples thereafter for 2 more days or in some instances for a longer period. In certain cases, especially in those of hyperthyroidism, the collections during the first few hours were made at 1- or 2- hour intervals. With regard to precision of collections of urine, a very satisfactory degree of cooperation has been achieved.

The urine was collected in glass bottles to which had been added sodium hydroxide (3 cc. of 2.5 normal solution) and sodium iodide (1 mgm.) as carrier. The specimens were carefully measured and 0.2 cc. was pipetted by means of a syringe pipette onto a copper planchette. Silver nitrate (0.1 mgm. silver in 0.1 cc.) was added to the drop on the planchette. If the sample was not properly centered on the planchette it was discarded and another was prepared. The specimen was then evaporated to dryness and counted at once with a Victoreen thin mica window beta-ray counter. The number of counts taken varied with the strength of the sample but, whenever possible, sufficient counts were made to keep the counting errors to within 2 to 5 per cent. All counts were made on duplicate samples. When these did not check closely the analysis was repeated on another pair of duplicates. The results were compared with a standard made from the same shipment of I^{131} as the tracer given the patient and were counted in the same manner and at the same time.

MATERIAL

Four groups of patients were studied. Group 1 included 4 normal men varying in age from 30 to 46 years, who served as controls. Group 2 consisted of 10 euthyroid patients who were given tracers because of the presence of papillary adenocarcinoma of the thyroid, graded 1 on a basis of 1 to 4 (Broders' method). So far as thyroid or renal function was concerned, these patients were essentially normal. Group 3 included 6 patients who had myxedema and in whom *in vivo* measurements failed to disclose any measurable collection of radioiodine by the thyroid. In 2 of the 6 cases the myxedema was adequately controlled by administration of desiccated thyroid at the time of this study. The remaining 4 patients were untreated at the time of this study. Group 4 included 16 patients who had uncomplicated, untreated exophthalmic goiter. None of these patients had received iodine before the study.

[1] Measurements made of aliquots of our samples in the laboratory of Dr. R. D. Evans, Massachusetts Intitute of Technology, indicated that the dose used closely approximated 200 microcuries, in terms of the standards of that laboratory. No attempt is made in this paper to discuss the problem of measurements of radioactivity indicated by this divergence of measurements.

RESULTS

Curves showing the cumulative urinary excretion of radioiodine by the subjects in the 4 groups mentioned are shown in Figures 1, 2, 3 and 4, respectively. The rate of excretion in the first 2 groups—the 4 normal men and the 10 euthyroid patients—varies somewhat but the curves are obviously exponential in form and each approaches a plateau in about 48 hours. The shape of the curves is strikingly similar to that of the curves constructed by Hamilton and Soley (13) from data obtained by *in vivo* measurement of radioactivity over normal thyroids after administration of radioiodine.

The rate of excretion of radioiodine by the myxedematous subjects is slower initially than that observed in the first 2 groups but persists much longer. A plateau may not be attained until after 4 days or more. Eventually, however, these subjects excrete in the urine much more of the administered dose of radioiodine than do the normal or euthyroid subjects.

The excretion of radioiodine by the patients who had untreated exophthalmic goiter exhibited a great deal of variation. This may not be surprising, since the severity of hyperthyroidism and the quantity of thyroid tissue present, as well as basal metabolic rates (range + 8 to + 82 per cent), varied greatly. Nevertheless the excretion rates of all but 2 of this group were well below the mean for normal persons. The 3 cases with highest excretion rates in this group are of particular interest because, although their response resembled that of the normals, they represent relatively severe exophthalmic goiter with basal metabolic rates of + 58, + 61 and + 46 per cent, respectively. The diagnosis was confirmed at operation in each instance. The excretion of radioiodine by the remaining subjects in this group was significantly less than in any of the other groups and the

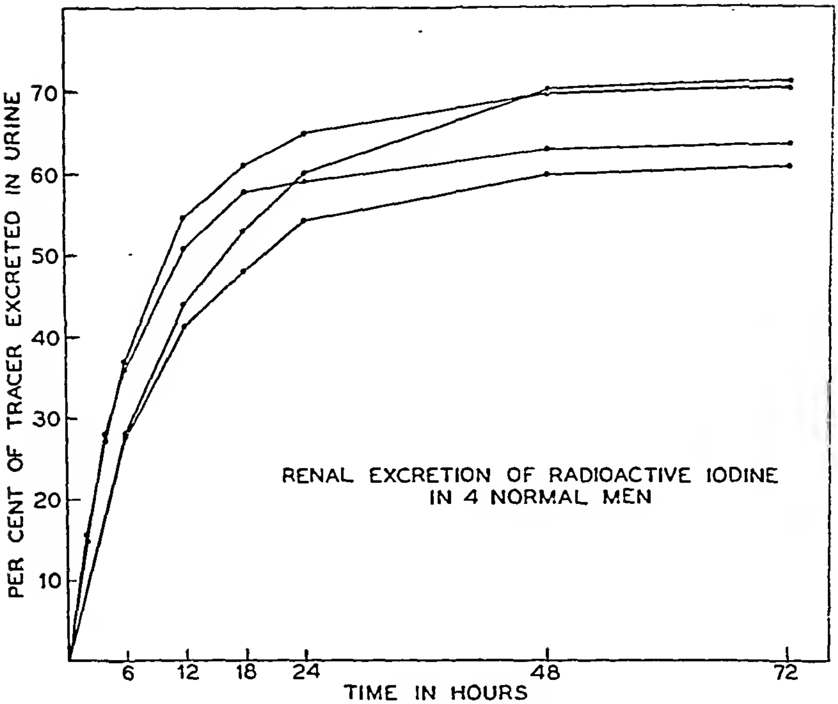

FIG. 1. URINARY EXCRETION OF RADIOIODINE IN 4 NORMAL MEN
The form of the curves suggests an exponential function.

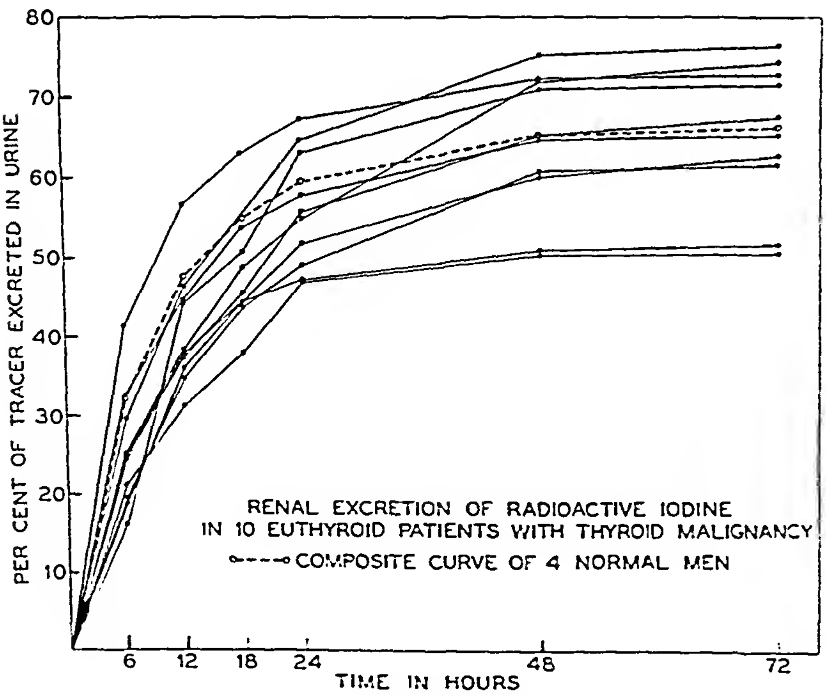

FIG. 2. URINARY EXCRETION OF RADIOIODINE IN 10 EUTHYROID PATIENTS WHO HAD LOW-GRADE THYROID MALIGNANT LESIONS

The composite curve of the normal men is included for comparison. Individual curves vary not only in the plateau which is reached but also in the time required to reach it.

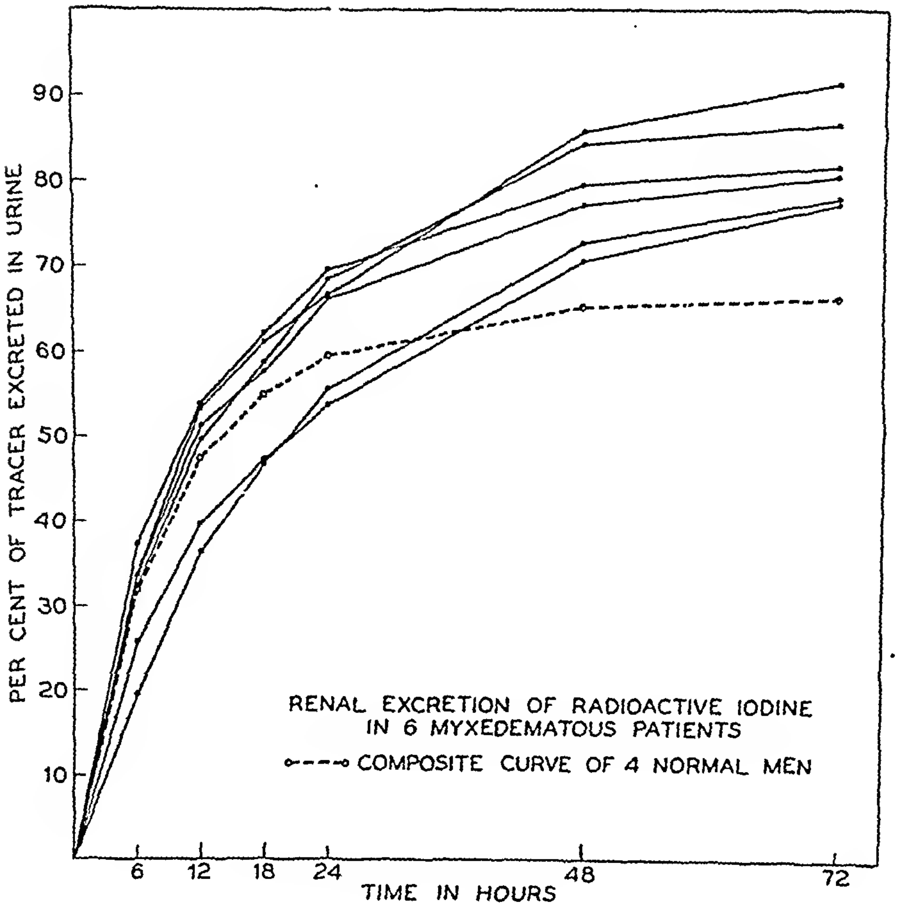

FIG. 3. URINARY EXCRETION OF RADIOIODINE IN 6 PATIENTS WHO HAD MYXEDEMA

The composite curve of the normal men is included for comparison. Not only does more radioiodine appear in the urine in myxedema but a much longer time is required to reach a plateau.

curves describing excretion appear to become asymptotic very quickly, especially in the instances of very low iodine output. Closer inspection of these curves, however, indicates that a real plateau is not reached; instead iodine excretion continues at a slow and relatively constant rate after the rather short initial phase of relatively rapid excretion is completed. In this sense the hyperthyroid curves appear therefore to be diphasic; there is an initial phase which, like that of the normal subjects, appears exponential and a second phase which is linear. As will be pointed out later, this difference from normals is more apparent than real; a linear phase is present in the normal curves also but is not apparent on casual inspection.

ANALYSIS OF DATA

The smoothness of the curves obtained, their exponential character and the variations encountered in the different groups suggested that the differences between them might be expressed quantitatively by mathematical analysis. A series of equations has been found which appears to accomplish this goal adequately.

The urinary curves are quite well represented by the function

$$Q = Q_f \ (1 - e^{-rt})$$

where Q is the amount of radioiodine, expressed as a fraction of the original dose, excreted in the urine in time t; Q_f is the asymptotic amount, that is, the total amount finally excreted, and r is a constant depending on the rate of disappearance of iodine from the blood. The function thus is seen to have 2 parameters which define it: r the rate constant, and Q_f the final value of Q. The value of r can be determined by plotting log $(Q_f - Q)$ against t (or the value of $Q_f - Q$ against t on

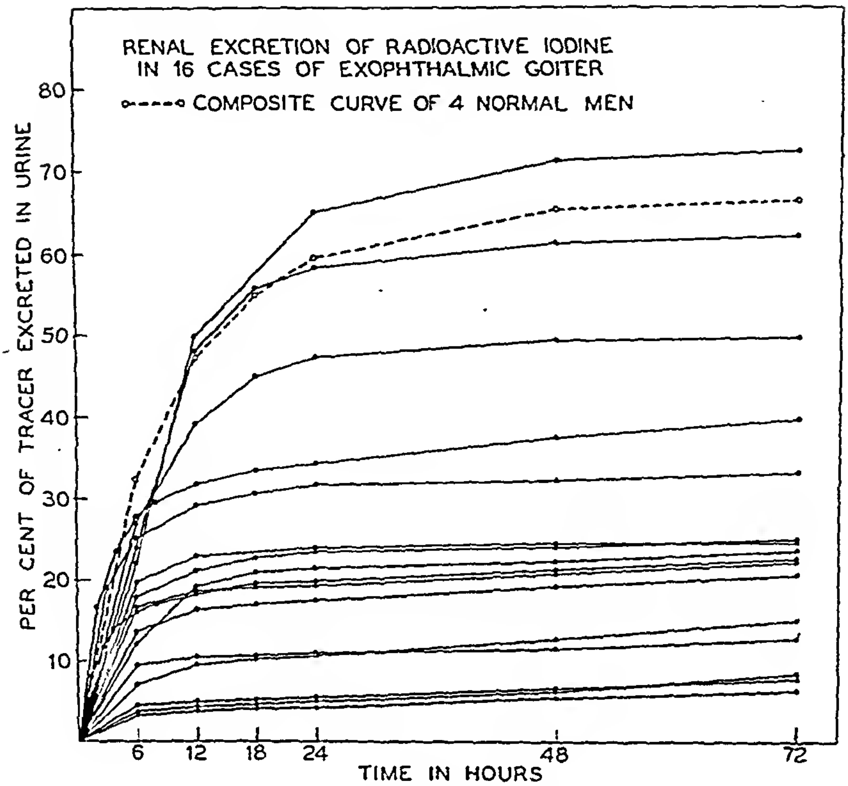

FIG. 4. URINARY EXCRETION OF RADIOIODINE IN 16 CASES OF UNTREATED EXOPHTHALMIC GOITER

The cases varied widely in clinical severity, basal metabolic rates and excretion of radioiodine. Instead of reaching a plateau, a slow and relatively constant excretion of radioiodine persists after the initial period of rapid excretion.

semilogarithmic paper) and determining the slope of the straight line function which results. This necessitates a prior knowledge of Q_f. The value of Q_f is estimated by inspection of the curve of the observed values Q versus t, and adjusted so as to give a linear function for log $(Q_f - Q)$ versus t.[2]

The proportional rate of removal of radioiodine from the blood by the kidneys and appearing as urinary radioiodine, is given by the product of r by Q_f. The value r itself is the proportional rate of disappearance of radioiodine from the blood into all tissues into which it goes. If therefore we assume for simplicity, and as a first approximation, that the iodine of the blood is going only to 2 tissues, the thyroid and the kidneys, the rate of absorption of iodine by the thyroid is given simply by the difference between the total rate and that estimated for the kidneys, that is, $r - rQ_f = r(1 - Q_f)$. This difference actually measures the rate of removal by all tissues together, other than the kidneys, and therefore, in so far as there are other tissues than thyroid involved, it does not give the true thyroid rate. However, inasmuch as variations in the disposal of iodine in the body to tissues other than the kidneys are likely to be due to variations in thyroidal absorption of iodine, the thyroid being the most important of these tissues, it seems reasonable to use the value $r(1 - Q_f)$ as an *index* of the rate of removal of iodine by the thyroid. When the radioiodine curve of the thyroid can be accurately determined by observations obtained from the subject's thyroid directly, the rate of collection by the thyroid can be estimated.

It was noted in the preliminary discussion of the results that the excretion curves of the hyperthyroid group were diphasic and included a sec-

[2] Detailed mathematical development and treatment will be given in a forthcoming publication.

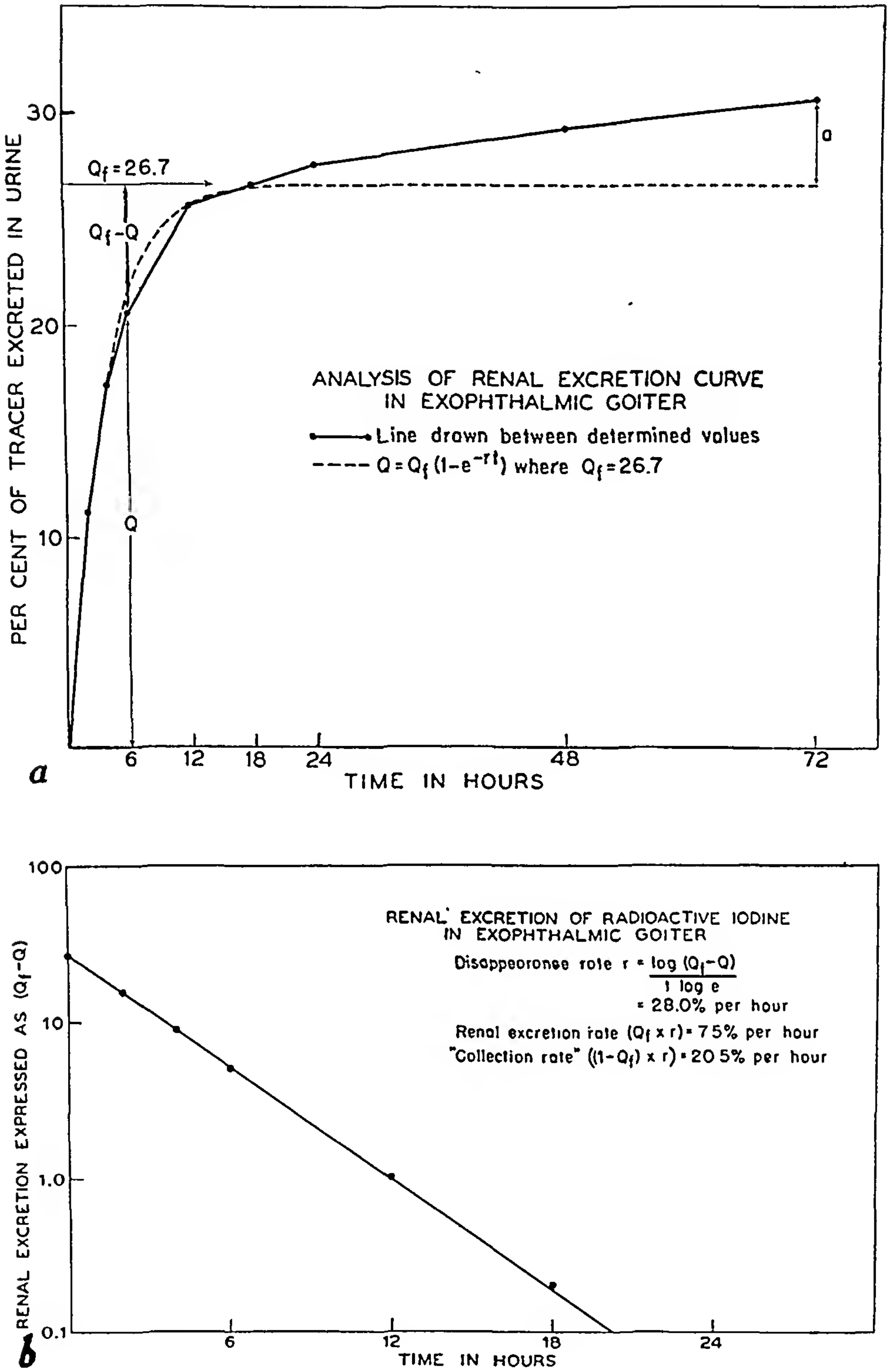

Fig. 7. Analysis of Urinary Excretion in a Case of Exophthalmic Goiter

a. The initial analysis of the curve shows clearly that, instead of a plateau, the initial phase of excretion is followed by a fairly constant or linear phase. When observations on euthyroid subjects are continued long enough, the same phenomenon is encountered. The difference between the actual excretion of radioiodine in 72 hours and that which is predicted from extrapolation of the initial excretion curve is called the quantity a for purposes of reference.

b. The initial renal excretion curve expressed as $Q_f - Q$.

5 or 6 days are required to approach it closely and since the proportion of the dose remaining is very small by that time, it cannot be concluded with certainty that this quantity is not present in these curves. However, it does not appear to be present and its apparent absence may prove both correct and significant.

It is a hypothesis that the excess excretion of radioiodine referred to in the previous paragraph is related indirectly to the secretion of radioiodine previously stored in the thyroid. Preliminary measurements of radioiodine in blood and preliminary *in vivo* measurements make it clear that this excess excretion in urine does *not* reflect *directly* the secretion of stored radioiodine by the thyroid. However, secretion of radioiodine by the thyroid is generally assumed to be largely in the form of organically bound iodine. Perhaps therefore one should not expect an immediate and direct reflection in urinary excretion.

Figure 5a and b shows the analysis of the renal excretion curve for a normal subject; Figure 6a and b, for a myxedematous one, and Figure 7a and b, for a case of exophthalmic goiter. Practically all of the cases studied fit the straight line as closely as the examples given and the fact that the data fit the mathematical assumptions so precisely lends some support to the validity of such treatment.

According to the mathematical analysis which leads to the function presented for the radioiodine curve, the value r, whether determined from the radioiodine curve of the urine or by any other method, measures the proportional rate of disappearance of radioiodine *from the blood*. Accordingly, it should be the same value however determined. Table I shows the results obtained in 4 cases in which a comparison has been made between the value for r determined from urinary data and the same quantity determined directly from blood samples, following the same tracer. The blood samples were drawn at intervals, digested with alkali and analyzed according to a technique which will be described elsewhere. The figures obtained from the 2 sources, blood and urine, agree very well. In Table II are values for disappearance rate, r, determined by 4 methods in a normal subject. The values for r were calculated from serial measurements over the thyroid *in vivo*, serial measurements of peripheral radioactivity over the thigh *in vivo*, serial blood samples and the renal excretion curve. These figures also agree reasonably satisfactorily when one considers that the measurement of radioiodine in blood is somewhat less accurate than in urine and that the *in vivo* measurements, in their present stage of development, are only close approximations.

TABLE I

Rate of disappearance of radioiodine

Case	Diagnosis	Rate of disappearance of radioiodine*	
		Urine	Blood
		per cent per hour	
1	Exophthalmic goiter	27.3	26.4
2	Exophthalmic goiter	32.6	31.4
3	Adenomatous goiter with hyperthyroidism	9.7	12.0
4	Adenomatous goiter with hyperthyroidism	12.9	12.4

* The quantity r, or disappearance rate, determined from urinary excretion curves compared with the rate determined from a series of blood samples obtained during the same period.

TABLE II

Rate of disappearance of radioiodine

Source of calculation	Rate of disappearance*
	per cent per hour
Radioiodine in urine	14.2
Radioiodine in blood	11.6
Radioactivity in thyroid, measured *in vivo*	10.2
Radioactivity in thigh, measured *in vivo*	11.9

* The quantity r, or disappearance rate, determined from 4 types of measurements in a normal subject following administration of a single tracer dose of radioiodine.

In Table III are given the means and standard deviations, for the 4 groups of cases comprising this study, of the following 4 quantities: (*1*) Q_f, which measures the fraction of radioiodine being excreted into the urine; (*2*) r, which measures the proportional rate of disappearance of radioiodine from the blood; (*3*) $r \times Q_f$, which measures the proportional rate of excretion into the urine and which will be referred to as the "renal excretion rate"; (*4*) $r\ (1 - Q_f)$, which measures the proportional rate of absorption by other tissues than the kidneys (thyroid?) and which will be referred to as the "collection rate." In addition, the mean

TABLE III

Summary of quantitative characteristics of renal excretion curves in various thyroid states

Group	Condition	Cases	Renal fraction (Q_f)	Disappearance rate (r)	Renal excretion rate ($r \times Q_f$)	Collection rate $r(1 - Q_f)$	Excess excretion in 72 hrs. (a)†
			per cent of dose	*per cent per hour*			*per cent of dose*
1	Normal conditions	4	65.2± 5.0*	11.1±3.6	7.2±2.1	3.9±2.8	1.4±1.4
2	Thyroid tumors (euthyroid)	10	65.1±11.0	8.9±2.1	5.7±1.8	3.2±1.7	0.6±0.5
3	Myxedema	6	84.6± 4.5	6.8±1.8	5.8±1.4	1.0±0.6	0
4	Exophthalmic goiter	16	24.8±22.2	28.9±5.1	5.4±2.0	23.5±8.0	2.4±1.4

* The mean of each group is given together with the standard deviation in order to indicate the relative variation encountered.

† The quantity a, which is also included, indicates the excess excretion of iodine in the urine after 72 hours. This expression does not attempt to define this factor quantitatively.

values of the excess excretion of radioiodine in urine, the quantity a, are included. The latter is expressed as the total excess after 72 hours. This is an admittedly inadequate means of expressing this quantity but it may serve until further study establishes its quantitative significance. The values as shown indicate the relative magnitude of the excess in the 4 groups of patients.

The variations in the renal fraction among the 4 groups of cases are what one would expect from inspection of the original excretion curves (Figures 1 to 4, inclusive). The normals excreted an average of 65 per cent, the myxedematous patients conspicuously larger fractions and the hyperthyroid patients conspicuously smaller and more variable quantities.

The values for disappearance rate, r, show larger differences than do the renal fractions. The values for hypothyroid subjects are significantly lower than for the normals and the values for hyperthyroid subjects are more than 2½ times as high as those for the normals.

The renal excretion rates ($r \times Q_f$) vary considerably from patient to patient and the variations are of sufficient order to account for some of the variations of iodine collection which are observed. However, no significant difference exists in the mean renal excretion rates of the 4 groups and all show approximately the same degree of individual variation. One might expect to find reduced renal excretion of significant degree in hypothyroidism. This point will have to be settled by study of a larger series of untreated patients.

The most striking differences are found in the values for collection rates, r $(1 - Q_f)$. The hypothyroid subjects show, as one would expect, exceedingly low collection rates, 0.6 to 1.6 per cent per hour. What proportion of this represents loss in feces and sweat, what proportion errors in the method, and what proportion actual collection by the thyroid or peripheral tissues it is impossible to say. The very high collection rates found in the cases of exophthalmic goiter may be regarded as due to variations of thyroid function, even though thyroid function is not here being measured directly. The mean collection rates in exophthalmic goiter were 6 times the normal rates whereas the renal fractions as such were less than half the normal values.

COMMENT

It is reasonable to assume that substantially all, if not quite all, of the radioiodine given as a tracer is either excreted in the urine or collected by the thyroid. The fraction of the total amount of iodine disappearing from the blood which is collected by the thyroid is therefore a relative quantity, depending on the function of the kidneys as well as the function of the thyroid itself. Even if the thyroid function with respect to iodine collection remains quite constant, a variation in function of the kidneys (or other iodine-collecting tissue) with respect to iodine collection will alter the picture of collection of iodine by the thyroid. Thus, if the function of the kidneys is altered so that the renal collection rate is lowered, the amount of iodine collected in any given time by the thyroid will increase, as will also the total amount of iodine eventually collected by the thyroid.

The following hypothetical case will illustrate

this point: If the thyroid-collecting rate was 3 per cent per hour and the renal excretion rate 5 per cent, the thyroid would collect in the first 24 hours 32 per cent of the original dose and asymptotically it would collect 37.5 per cent of the dose. If now the thyroid rate remained at 3 per cent but the renal excretion rate was reduced to 1 per cent, the thyroid would collect in the first 24 hours 46.1 per cent of the original dose and asymptotically 75.0 per cent. Attention only to the amounts of iodine collected by the thyroid might therefore easily give the impression that the thyroid function had been altered. Use of the formulae presented earlier in this paper would avoid this erroneous conclusion, for what remains constant in the situation of unaltered thyroid function is the product of r and Q_f, which for the situation before the alteration of the renal function would be $0.375 \times 0.08 = 0.03$ and after the alteration of the renal function would still be $0.75 \times 0.04 = 0.03$. The method of study which has been described suggests a means for determining the capacity of the kidneys to excrete iodine without regard to thyroid function and of the thyroid to collect iodine without regard to variations of renal function.

Tracers are ordinarily considered as labeling a specific quantity of iodine so as to make its behavior distinct from that of the material with which it is mixed. It is also true, however, that once a radioiodine tracer is mixed with the circulating *iodide* of the blood (either after complete absorption of an oral dose or after intravenous administration) it may be regarded as a label temporarily, not only of the tracer dose, but also of all of the *iodide* in circulation in the blood and in the body fluids which are in osmotic equilibrium with the blood. In this sense whatever happens to the tracer is a valid quantitative measure of what is happening to the circulating iodide, since the two behave identically. The quantities which have been proposed, therefore, express the percentage of the total circulating blood iodide which is being excreted or otherwise disposed of, as well as the percentage of the tracer which is disappearing. This does not necessarily imply that the circulating iodide in the blood is constant in amount. It merely implies that whatever the range of variation of circulating *iodide* in blood at the time of observation, the renal excretion rate and the collection rate (expressed as a percentage of the total quantity) are relatively constant quantities.

The radioiodine tracers which have been studied previously, as well as those described in the present study, have all been given orally. This raises the question whether the time required for absorption affects the results significantly. Preliminary observations suggest that absorption time has not been a critical factor in the majority of instances. However, a comparative study of intravenous versus oral tracers is in progress in order to elucidate this point more fully.

Hamilton, (18), in an early paper, described the remarkable speed with which an oral dose of radioiodine appeared in the blood. Using a Geiger-Müller counter over the hand as an indicator, he found the absorption was 80 per cent complete in an hour. In some preliminary studies we have found that in both normal and hyperthyroid subjects, absorption may be complete in an hour or less. Because the absorption from the gastro-intestinal tract is complete within such a short period it appears reasonable at the moment to assume that, in most instances, only a small error will result from ignoring it. There is some suggestion that the presence of food in the stomach may somewhat prolong absorption of radioiodine and on this account we have made it a practice to give tracers to patients in the fasting state.

It is obvious that sooner or later the iodine collected and stored in the thyroid is likely to be secreted from the gland. If one excepts the observations made by Hamilton and Soley and their associates (14) of the behavior of very large doses of labeled iodide in toxic goiters and in hypothyroid children with goiter, subsequent secretion of radioiodine by the thyroid appears to occur much more slowly than its initial collection. So long as it takes place relatively slowly, thyroidal secretion will not modify the exponential character of the initial collection or excretion curves until either is approaching an asymptotic level. Studies of the quantitative character of the subsequent secretion of radioiodine by the thyroid may as a matter of fact prove far more significant than collection studies. However, because of artefacts resulting from the quantity of radiation in the

tracer, they may be subject to more uncertainty than are the studies of collection rates.

The quantity of radiation employed in all tracer studies which have so far been reported substantially exceeds in the thyroid the arbitrary tolerance dose of 0.1 roentgen per day which is approved for total body radiation of persons working with radium or roentgen rays. Considering the great concentrating power of the thyroid, doses of I^{131} of the order of 1 or 2 microcuries would have to be used in order to keep the radiation exposure of the thyroid below 0.1 equivalent roentgen per day. Such minute tracers are not practicable with present methods. The radiation dosage in the present study, assuming the dose employed to be 200 microcuries of I^{131}, is of the order of 20 equivalent roentgens per day or a total integrated dose per patient of around 230 equivalent roentgens. No information is available regarding the minimal quantity of radiation per day which is capable of inducing biologic effects in the thyroid either within a few hours or within a period defined by a few half lives of I^{131}. Until this is known the possibility must always be considered that the radiation resulting from tracer doses of the magnitude employed may produce biologic effects which influence the results observed.

Another factor of importance in the evaluation of tracer studies is the quantity of carrier, or inert iodide, which is given with the radioiodine. The comparison by Hamilton and Soley of the difference in response to a 14-mgm. dose of sodium iodide compared with a dose of 0.1 microgram leaves no doubt that the size of the carrier can modify the result obtained. For most purposes it is desirable that the quantity given in a tracer be an insignificant amount or at most a small increment to the iodide already present in the body. We have so far avoided very small tracers (1.0 microgram or less) because of the fear, perhaps unfounded, that excessive losses will occur with such tiny quantities because of adherence to containers and so forth. If one assumes blood iodide to be in osmotic equilibrium with the extracellular fluid, as it probably is, the carrier which we have employed, 100 micrograms, should represent an average increment of the order of 1 microgram of sodium iodide per 100 cc. of plasma. Before it can be said with certainty that this is an increment which does not of itself modify iodine metabolism more detailed studies with varying quantities of carrier will be needed.

SUMMARY

The urinary excretion of radioiodine following its oral administration has been studied in normal subjects, euthyroid patients who had low-grade thyroid malignant lesions, patients who had myxedema and patients who had untreated exophthalmic goiter.

The urinary excretion curves differ significantly among the euthyroid, hypothyroid and hyperthyroid groups. Mathematical analysis of the urinary excretion yields the following 4 quantities: (*1*) the renal fraction (which is the fraction of the dose of radioiodine primarily excreted in the urine); (*2*) the disappearance rate (which is the proportional rate of disappearance of radioiodine from the blood); (*3*) the renal excretion rate (which is the proportional rate of excretion into the urine); and (*4*) the collection rate (which measures the proportional rate of disappearance into other sites than the kidneys). In so far as the thyroid represents the chief site in the body for collecting iodine, the last of these 4 values serves as an index of thyroid-collection rate. When the radioiodine curve of the thyroid can be determined by observations obtained from the subject's thyroid directly, the true rate of collection by the thyroid can be estimated.

Estimations of disappearance rate, based on analysis of renal excretion, have been found to agree fairly closely with estimations of the same quantity based on direct measurements of blood samples, measurements of the thyroid *in vivo* and measurements of peripheral radioactivity *in vivo*.

The renal fraction is greater than normal in hypothyroid patients and is less than normal in hyperthyroid patients. The disappearance rate is less in hypothyroid patients than in normal subjects and is markedly greater than normal in cases of hyperthyroidism. Significant individual variations occur in renal excretion rates but no significant differences were observed in the mean renal excretion rates among the 4 groups of cases studied. The most significant variation observed occurred in the collection rates, which were very much less than normal in the hypothyroid cases,

and averaged 6 times the normal in the hyperthyroid patients.

The significance, the limitations and the possibilities of this technique have been discussed.

BIBLIOGRAPHY

1. Hertz, S., Roberts, A., and Evans, R. D., Radioactive iodine as indicator in study of thyroid physiology. Proc. Soc. Exper. Biol. & Med., 1938, 38, 510.
2. Hertz, S., Radioactive iodine as an indicator in thyroid physiology; observations on rabbits and on goiter patients. Am. J. Roentgenol., 1941, 46, 467.
3. Hertz, S., Roberts, A., and Salter, W. T., Radioactive iodine as an indicator in thyroid physiology. IV. The metabolism of iodine in Graves' disease. J. Clin. Invest., 1942, 21, 25.
4. Keating, F. R., Jr., Rawson, R. W., Peacock, W., and Evans, R. D., The collection and loss of radioactive iodine compared with the anatomic changes induced in the thyroid of the chick by the injection of thyrotropic hormone. Endocrinology, 1945, 36, 137.
5. Larson, R. A., Keating, F. R., Jr., Peacock, W., and Rawson, R. W., The effect of thiouracil on the collection of radioactive iodine by the thyroid of the chick. Endocrinology, 1945, 36, 160.
6. Leblond, C. P., and Süe, P., Iodine fixation in the thyroid as influenced by the hypophysis and other factors. Am. J. Physiol., 1941, 134, 549.
7. Leblond, C. P., Fertman, M. B., Puppel, I. D., and Curtis, G. M., Radioiodine autography in studies of human goiterous thyroid glands. Arch. Path. 1946, 41, 510.
8. Leiter, L., Seidlin, S. M., Marinelli, L. D., and Baumann, E. J., Adenocarcinoma of the thyroid with hyperthyroidism and functional metastases. I. Studies with thiouracil and radio-iodine. J. Clin. Endocrinol., 1946, 6, 247.
9. Perlman, I., Morton, M. E., and Chaikoff, I. L., Radioactive iodine as an indicator of the metabolism of iodine. II. The rates of formation of thyroxine and diiodotyrosine by the intact normal thyroid gland. J. Biol. Chem., 1941, 139, 449.
10. Franklin, A. L., Chaikoff, I. L., and Lerner, S. R., The influence of goitrogenic substances on the conversion *in vitro of* inorganic iodide to thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. J. Biol. Chem., 1944, 153, 151.
11. Rawson, R. W., Tannheimer, J. F., and Peacock, W., Uptake of radioactive iodine by thyroids of rats made goiterous by potassium thiocyanate and by thiouracil. Endocrinology, 1944, 34, 245.
12. Hamilton, J. G., and Soley, M. H., Studies in iodine metabolism by the use of a new radioactive isotope of iodine. Am. J. Physiol., 1939, 127, 557.
13. Hamilton, J. G., and Soley, M. H., Studies in iodine metabolism of thyroid gland in situ by use of radio-iodine in normal subjects and in patients with various types of goiter. Am. J. Physiol., 1940, 131, 135.
14. Hamilton, J. G., Soley, M. H., Reilly, W. A., and Eichorn, K. B., Radioactive iodine studies in childhood hypothyroidism. Am. J. Dis. Child., 1943, 66, 495.
15. Rawson, R. W., Hertz, S., and Means, J. H., Thiocyanate goiter in man. Ann. Int. Med., 1943, 19, 829.
16. Rawson, R. W., Evans, R. D., Means, J. H., Peacock, W. C., Lerman, J., and Cortell, R. E., The action of thiouracil upon the thyroid gland in Graves' disease. J. Clin. Endocrinol., 1944, 4, 1.
17. Seidlin, S. M., Marinelli, L. D., and Oshry, Eleanor, Radioactive iodine therapy; effect on functioning metastases of adenocarcinoma of the thyroid. J. A. M. A., 1946, 132, 838.
18. Hamilton, J. G., The rates of absorption of the radioactive isotopes of sodium, potassium, chlorine, bromine, and iodine in normal human subjects. Am. J. Physiol., 1938, 124, 667.

STUDIES ON PAIN: DISCRIMINATION OF DIFFERENCES IN INTENSITY OF A PAIN STIMULUS AS A BASIS OF A SCALE OF PAIN INTENSITY

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INTRODUCTION

In the century which has elapsed since Weber first pointed out that the ability to discriminate just noticeable differences in 2 stimuli inducing sensation depends upon the magnitude of the stimulus, psychologists and physiologists have made use of this relationship for the study of nearly all types of sensation (1). Pain has not been so studied, perhaps because of the view formerly held that pain was not a sensory entity but was the end-point of over-stimulation of any of the recognized sensory mechanisms of the body (2). Recent evidence, however, makes it appear likely that pain per se is a sensation with specific sensory apparatus in the skin and deeper tissues, and with its own neural pathways and functional properties (3).

Investigation of the Weber ratio ($\Delta I/I$, in which I = intensity) has lead to at least 3 useful formulations about sensations other than pain.

1. The range of effective intensities of the adequate stimulus has been determined. For example, the range of effective stimuli for vision between threshold stimulus and the "dazzle" point is approximately 10 billion fold (4). For warmth sense, this range from threshold to onset of thermal pain is about 2 thousand fold (5).

2. The number of just noticeable differences which the average individual can distinguish in the range of effective stimuli has also been ascertained. Thus, there are approximately 570 barely distinguishable steps for vision in the range of intensity from complete darkness to the dazzle point (6) and about 90 steps between the warmth threshold and the thermal pain threshold (7).

3. It has been suggested that large changes in the numerical value of the Weber ratio are associated with changes in the quality of sensation. As an example may be cited the attempt to correlate the change in $\Delta I/I$ at about 0.02 millilambert with the change from achromatic to chromatic vision (8).

The Weber Law states simply that as the intensity of a stimulus increases, the ability to discriminate differences in intensity of stimulus decreases proportionately. For a stimulus of intensity I, there is a minimum change in intensity, ΔI, which can just barely be detected when added to or subtracted from I. That is, $I \pm \Delta I$ can just be distinguished from I, and ΔI is the "just noticeable difference" (JND) in intensity of stimulus, or the difference limen (dl). Algebraically the Weber Law can be written: $\Delta I/I = C$, in which $\Delta I/I$ is known as the Weber ratio and C is a constant. This relationship has been shown to be valid over limited ranges of stimulus intensity for vision, hearing, and temperature sensation (7). It was Fechner who suggested that the constant in the equation could be interpreted as proportional to the minimal difference in sensation (ΔS) and that:

$C = K\Delta S$, in which K is a proportionality factor and ΔS is the just noticeable difference in sensation.

Therefore, $\Delta I/I = K\Delta S$, or upon integration,

$S = K \log I/I_0$, where: S = intensity of sensation evoked by the stimulus of intensity I, I_0 = threshold stimulus intensity.

The importance of this conception lies mainly in the fact that it has afforded another quantitative approach to the study of sensations. It is the purpose of this communication to present the results of measurements of the Weber ratio for cutaneous pain sensation, and to suggest the usefulness of these measurements as a basis for the evaluation of the intensity of pain sensation.

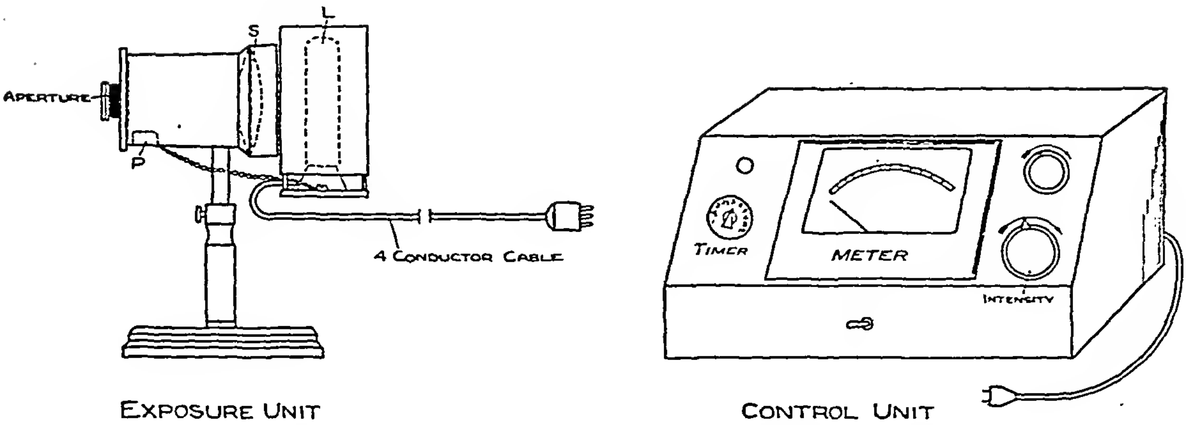

FIG. 1. SCHEMATIC DRAWING OF THE SIMPLIFIED PAIN THRESHOLD EQUIPMENT
L, 500 watt lamp; S, condensing lens of 20 cm. focus; P, shutter relay.

METHOD

The painful sensation which was studied in these experiments was induced in the skin by a 3-second exposure to intense heat radiation. A modified Hardy-Wolff-Goodell pain threshold equipment was employed as the stimulator (5). The apparatus has been altered from that originally described by replacing the rheostat with a variac transformer to alter the intensity of the light beam, and the use of a sensitive voltmeter especially adapted to indicate the intensity of the radiation in millicalories per second per cm.^{2} (0.001 gram cal./sec./cm.^{2}). The reading of the voltmeter was checked from time to time with a calibrated radiometer. This is an essential procedure for an apparatus which depends upon the measurement of electrical input to the heat source. An electronically controlled shutter gave considerable flexibility to the equipment as it was no longer necessary to employ a pendulum to limit the time of exposure. As the changes which have been incorporated give increased flexibility and make possible a greater variety of experiments, a schematic drawing of the simplified apparatus is shown in Figure 1.[1]

The method of measuring the just noticeable differences (ΔI) for the pain induced by heating the skin with radiant energy was as follows: An intensity of radiation (at or [illegible] than the pain threshold) was selected each experimental day as the "standard" for that experiment. The method of choosing the standard was to increase systematically the intensity of the stimulus by approximately the amount of ΔI as determined from the previous experiment. A series of 13 standard stimuli were used, including 220 millicalories (approximately the pain threshold). The 3 authors, serving in turn as subject and observer, were each stimulated with 2 exposures to the standard radiation. The forehead, blackened with India ink, was used as the test surface because of its uniform temperature and because this area had served satisfactorily in the past for pain threshold studies. In the series of experiments with stimuli greater than 500 millical./sec./cm.^{2}, considerable tissue damage was produced. For this reason, a second test area, the blackened volar surface of the forearm, was chosen. This area had the same pain threshold as the forehead and was more easily cared for when blistered. Following exposure to the standard, the subjects were presented, in rotation, with 3 test stimuli. The sensation induced by the standard stimulus was compared from memory with the sensations evoked by the test stimuli and a report made as to whether the test stimuli were equal to, less than, or more than the standard. It required about 10 minutes to present the test stimuli and record the reports, as it was important not to irradiate the skin in too rapid succession. Intervals of less than 1 minute between stimulations were found to introduce uncertainty due to after-sensations. Following the first series of 3 test stimuli, the standard was again presented, the subject being so informed, and a second series of tests begun. This procedure was followed with increasing and decreasing intensities of stimulus until it became evident that ΔI had been ascertained. ΔI was established as the intensity difference which the subject recognized in 2 out of 3 trials.

A high degree of attention on the part of the subject was required to obtain uniform results and, in some instances, the experiment had to be delayed because of the temporary inability of a subject to concentrate sufficiently. Some improvement in discrimination was observed in the subjects as they became accustomed to the experimental procedure. This was apparent mainly in more uniform results with fewer wide variations. The subjects were agreed that this experiment required much more in the way of concentration and attention than did measurements of pain thresholds and, for this reason, conversation and interruptions during an experiment were avoided.

[1] Changes from the original design were made by the Experimental Engineering Corporation, Bergenfield, New Jersey.

RESULTS

The results of the experiment are contained in Table I. Each value of ΔI reported in column 2 of Table I represents an average of 3 or more observations on 3 individuals.

TABLE I

Average values of ΔI and the Weber ratio for pain sensation

Standard stimulus intensities	Average ΔI	Average $\frac{\Delta I}{I}$
	millicalories/second/cm.²	
222	7	.03
240	7	.03
258	8	.03
270	6	.02
291	6	.02
300	11	.04
312	9	.03
330	15	.05
354	19	.06
366	16	.04
390	17	.04
420	29	.07
480	60	.13
680	200	.29
(1100)	(620)	(.56)

In the range of stimuli from threshold to 420 millical./sec./cm.², individual determinations of ΔI usually deviated from the average value by approximately ± 20 per cent. At the higher intensities, because of the damage done to the skin by the intense stimuli, just noticeable differences could not be so carefully ascertained, and a variation of as much as ± 50 per cent resulted.

The relationship between the intensity of the stimulus and the "just noticeable difference" is shown in Figure 2. At threshold, ΔI is approximately 7 millicalories, or ± 3 per cent of the threshold. This value of ΔI corresponds quite well with the observations previously reported on the preciseness with which the threshold can be measured by this method, that is ± 4 per cent. There is no apparent increase in the value of ΔI between the threshold stimulus and 290 millical./sec./cm.², but between this latter intensity and 340 millical./sec./cm.², ΔI more than doubles, attaining the value of 15 millical./sec./cm.². Between 340 and 400 millical./sec./cm.² there is little change in ΔI, but beginning at about this latter intensity, there is a final sharp rise in the ΔI values.

Thus, although 420 and 480 millical./sec./cm.² can be distinguished, 480 and 580 millical./sec./cm.² cannot, and it is not until the stimulus has been increased to 680 millical./sec./cm.² that a barely perceptible difference can be noted. In tests on 5 subjects, 3 subjects reported 680 as more intense than 480 millical./sec./cm.² and 2 subjects reported the sensations as indistinguishable. In 2 experiments, 2 subjects reported pain intensity evoked by 1100 as definitely more intense than that induced by 480 millical./sec./cm.² One subject reported 680 and 1100 millical./sec./cm.² as indistinguishable. Thus discrimination is such for stimuli greater than 680 millical./sec./cm.² that, although it is possible to distinguish these stimuli from 480 millical./sec./cm.², it is not possible to make a distinction between these high stimuli themselves. The sensation evoked by a stimulus of about 680 millical./sec./cm.² is therefore a "ceiling" pain since stimuli of greater intensity cause no perceptibly greater pain. Beyond the ceiling pain, ΔI increases by the amount the intensity of the stimulus is raised above that causing the ceiling pain. For example, ΔI for ceiling pain is − 200 millical./sec./cm.², ΔI for 1100 millical./sec./cm.² is − (200 + 420) millical./sec./cm.², the latter quantity being simply the difference between 1100 millical./sec./cm.² and the ceiling stimulus intensity. In the intensity range above 400 millical./sec./cm.², ΔI is changing so rapidly that the discrimination for higher intensities is considerably less than that for lower intensities. For example, 420 can be distinguished from 390 with a ΔI of 30 millical./sec./cm.² whereas 420 can barely be distinguished from 480 with a ΔI of 60 millical./sec./cm.².

DISCUSSION

a. The stimulus

It is probable that the magnitude of the ΔI and its ratio to the intensity I will depend to a large extent upon the type of stimulus used, and it is to be expected that measurements made with electrical and other types of stimulus will not necessarily correspond with the values reported here. It is desirable that the stimulus chosen be one for which the quantity measured is proportional to the amount of pain producing activity at the pain endings, that is, the algesic action of the stimulus. This is of importance because measurements of

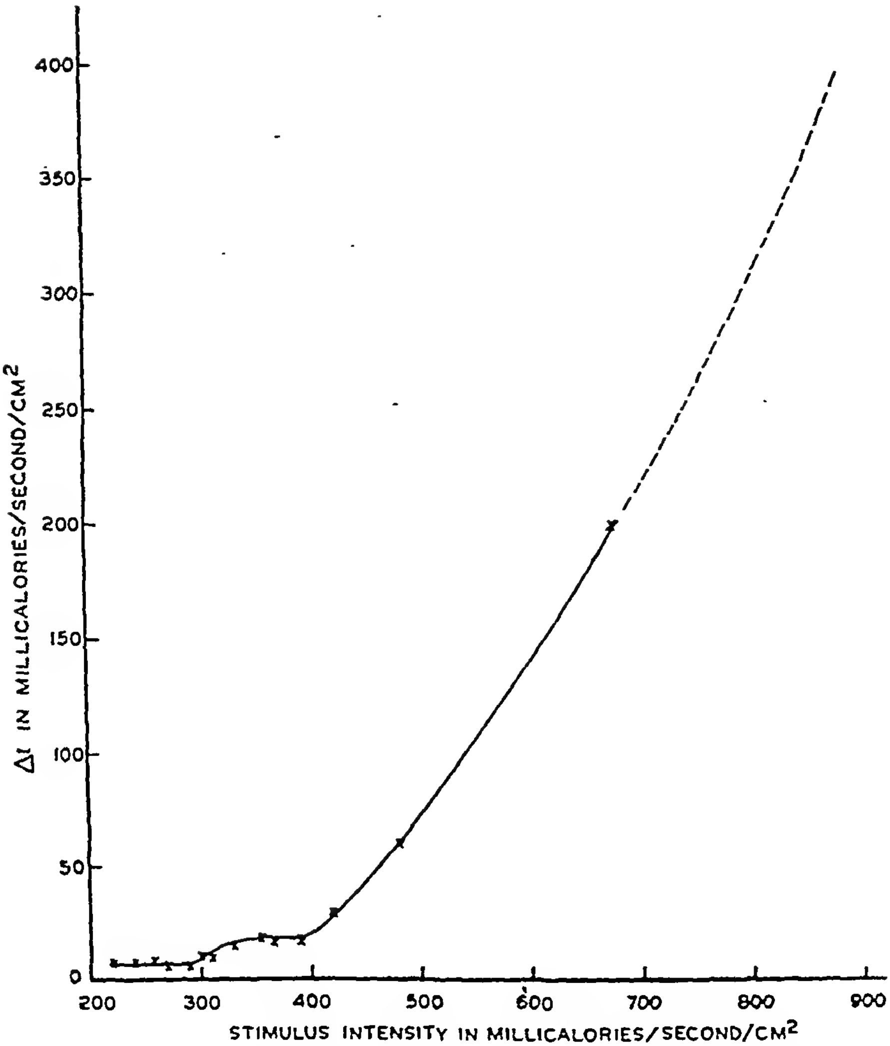

FIG. 2. ΔI FOR INTENSITIES OF THERMAL RADIATION INDUCING CUTANEOUS PAIN

ΔI with an indirect type of stimulus, such as the electrical stimulation of the tooth, may require a different interpretation. For example, the direct electrical stimulation of a nerve fiber subserving pain should yield a series of ΔIs which are dependent upon the condition of the nerve fiber and its electrical relation to the stimulating electrode. In the usual investigations of sensation, attention has been rightly focused on the "adequate" stimulus and, as radiant heat stimuli affect pain endings in a physiological manner, it is likely that thermal radiation is an adequate stimulus for producing pain sensation in the skin. It is apparent from Table I that by means of a suitable stimulus, reproducible measurements can be made of the just noticeable difference of painful stimuli.

The painful sensation caused by the highest stimulus intensities is distinctly different from the pain experienced in the stimulus range below 480 millical./sec./cm.². After the greater stimuli, the subjects reported a deep aching quality as well as the bright burning quality during the 3-second exposure. At the termination of the exposure, the deep aching pain persisted at a high intensity for some seconds as an after-sensation. For the weaker stimuli, the after-sensation had a low intensity burning quality which was generally not observed after a strong stimulus. This difference in the after-sensations may be due to the functional elimination by damage of most of the superficial endings, and to the stimulation of the deeper pain endings. The most intense stimuli

evoke the ceiling pain in about ½ second and the level of pain does not increase during the exposure time, whereas the weaker stimuli evoke sensations which build up during the exposure. This experience supports the idea that stimuli greater than 680 millical./sec./cm.^{2} evoke the "ceiling" pain.

The reproducibility in any given individual of the amount and nature of the damage caused in the skin by the thermal radiation suggests the possible usefulness of this technique in the production of experimental lesions.. For example, on the skin of the volar surface of the forearm, stimuli of 400 to 480 millical./sec./cm.^{2} caused erythema; those of 500 to 700 millical./sec./cm.^{2} caused bleb formation, whereas those of approximately 1000 millical./sec./cm.^{2} caused necrosis without obvious bleb formation except at the margins of the exposed area.

b. The Weber ratio for pain

The value of $\Delta I/I$ is approximately constant in the range of stimuli from threshold to about 320 millical./sec./cm.^{2}, beyond which intensity there is an increase in the Weber ratio. An interpretation of Figure 2 may be made on a basis similar to that for evaluations of the Weber Ratio for stimuli producing other types of sensation. That is, the range of intensities between 220 and 320 millical./sec./cm.^{2} represents the stimulation of a type of cutaneous pain for which $\Delta I/I$ = constant = 0.03. At about 320 millical./sec./cm.^{2} another sensory element may have been added to the total sensory experience. This agrees with the experience that the quality of the pain sensation changes at about this point from a definite pricking sensation to one with an added burning quality. Indeed, this particular sensation has been chosen by 1 investigator as the pain threshold sensation rather than the sensation of minimal pricking pain which begins at about 220 millical./sec./cm.^{2} for a 3-second exposure (9).

c. A scale of pain intensity

That pain has the property of intensity is well recognized. Estimates of the intensity of painful sensations have been made commonly in terms such as severe, moderate or mild, and, in the past, the judgments of the subject have been useful in experimental and clinical procedures. Although it is not possible to say that the sensation induced by a given painful stimulus in one individual will be exactly reproduced, under apparently the same experimental conditions, in another individual, on the basis of the observed predictability of behavior and of report this appears to be quite likely. Also, useful scales have been devised for other sensations, such as the scale of visual brightness and the scale of the loudness of sound (10). Therefore, on the basis of the predictability of sensory phenomena in general, we might expect to set up a sensory scale of pain and, from the data presented in Table I, we have made such an attempt.

The stimulus intensity which gives rise to the threshold sensation marks the lower limit of the range of effective stimuli. The threshold sensation is arbitrarily assigned the value 0, meaning simply the beginning of the sensory scale. A stimulus which induces the ceiling pain determines the upper limit of the scale.

TABLE II
Scale of pain intensity

Stimulus intensity	Size of step	Number of steps	Number of dols	Amount of stimulus above threshold
millicalories/second/cm.^{2}				*millical./sec./cm.^{2}*
220			0	0
227	7	1		
234	7	2	1	14
241	7	3		
248	7	4	2	28
255	7	5		
262	7	6	3	42
269	7	7		
276	7	8	4	56
283	7	9		
290	7	10	5	70
300	10	11		
310	10	12	6	90
320	10	13		
335	15	14	7	115
350	15	15		
365	15	16	8	145
380	15	17		
395	15	18	9	175
425	30	19		
480	55	20	10	265
680	200	21		

From Table II, it can be seen that there are 21 barely perceptible steps between the threshold sensation and the ceiling pain. Except under the best experimental circumstances, it is not possible to distinguish stimuli closer together than 2 steps,

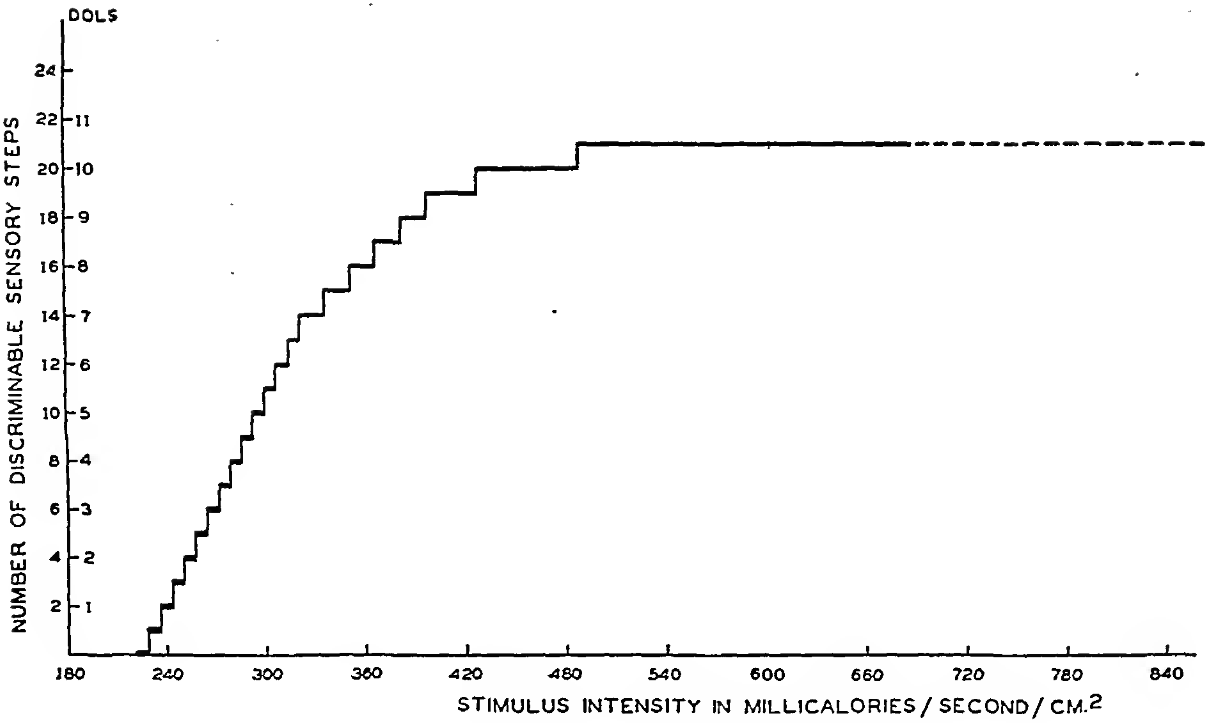

FIG. 3. GRAPHIC REPRESENTATION OF SCALE OF PAIN INTENSITY

and for this practical reason, the unit for pain sensation has been chosen as equivalent to 2 steps. It is suggested that the term "dol" be used to signify this unit. The ceiling pain intensity will therefore have the value of 10½ dols, as there are 21 barely discriminable steps in sensation between the pain threshold and the ceiling pain under our experimental conditions. Fortunately, as the threshold stimulus on the forehead and on the forearm is generally very near 220 millical./sec./cm.^{2}, little correction is required of the scale in Table II when these skin areas are used. A sensory scale of pain, such as that presented in Table II, is in keeping with the assumption of Fechner that the intensity of sensation evoked by a given stimulus is equivalent to the number of the discriminable steps from the threshold sensation. Figure 3 is a graphic representation of the relationship between the stimulus intensity, number of discriminable steps, and the dol scale of pain. The scale as represented in Figure 3 refers, of course, only to skin areas which have a pain threshold at 220 millical./sec./cm.^{2}.

There are 2 advantages in setting up a scale of pain sensation on the basis of the present data, namely: (*1*) the sensory range is clearly defined by the threshold sensation and the maximal possible, or ceiling pain sensation, and (*2*) the intensity of the pain can be related to an easily controlled and reproducible adequate stimulus. The usefulness of such a scale for pain sensation is further indicated by studies which will be reported in detail elsewhere. It permits the quantitative estimation of pain intensity above the pain threshold, for instance, in the study of the nature of hyperalgesia and the effect of local and general analgesics. It also provides a useful reference scale of pain intensity against which can be made quantitative estimates of the intensities of spontaneously occurring pain in disease.

SUMMARY AND CONCLUSION

1. Measurements of just noticeable differences in estimation of painful stimuli have been made with the Hardy-Wolff-Goodell pain threshold equipment by inducing pain in the skin with thermal radiation.

2. The effective range of this stimulus is limited by the pain threshold and pain of maximal intensity.

3. Pain induced in the skin by thermal radiation has a ceiling intensity and this ceiling pain was produced on the forearm by a stimulus intensity of 680 millical./sec./cm.^{2} in a 3-second exposure.

4. The Weber ratio for pain is approximately constant between threshold and about 320 millical./sec./cm.^{2}. An increase in the ratio at this point suggests that an additional sensory entity with a different quality of pain has been stimulated.

5. Twenty-one discriminable intensities of pain were observed between the threshold pain and the ceiling pain.

6. On the basis of the evidence presented above, a scale of pain intensity is proposed, the unit of which is called a "dol," composed of 2 just perceptible steps in discrimination of stimulus intensity.

BIBLIOGRAPHY

1. Culler, E. A., Thermal discrimination and Weber's Law. Arch. of Psychol., 1926, 13, 81.
2. Dallenbach, K. M., Pain: History and present status. Am. J. Psychol., 1939, 52, 331.
3. Tower, S., Pain: Definition and properties of the unit for sensory reception. A. Research Nerv. and Ment. Dis., Proc., 1943, 23, 16.
4. Hecht, S., The nature of the visual process. Harvey Lectures, 1937.
5. Hardy, J. D., Wolff, H. G., and Goodell, H., Studies on pain: A new method for measuring pain threshold: Observations on spatial summation of pain. J. Clin. Invest., 1940, 19, 649.
6. Best, C. H., and Taylor, N. B., Physiological Basis of Medical Practice. Williams and Wilkins Co., Baltimore, 1943, p. 1628.
7. Herget, C. M., Granath, L. P., and Hardy, J. D., Thermal sensation and discrimination in relation to intensity of stimulus. Am. J. Physiol., 1941, 134, 645.
8. Hecht, S., Rods, cones, and the chemical basis of vision. Physiol. Rev., 1937, 17, 239.
9. Chapman, W. P., and Jones, C. M., Variations in cutaneous and visceral pain sensitivity in normal subjects. J. Clin. Invest., 1944, 23, 81.
10. Stevens, S. S., and Davis, H., Hearing. John Wiley & Son, New York, 1938.

THE EFFECT OF URINE VOLUME ON UREA EXCRETION

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In recent papers Bing (1) and Williams (2) have proposed formulae which these authors believe to be more accurate than the original "standard" and "maximum" clearance equations of Möller, McIntosh, and Van Slyke (3, 4), in expressing the effects of urine volume flow on the urea clearance in human subjects. Both Bing and Williams base their formulae on the data of Möller *et al* (3).

The essential test of accuracy of such a formula is the consistency with which it permits one to calculate, from clearances shown by a subject with widely varying urine flows, the clearance that he would show with a given constant urine flow. Neither Williams nor Bing has applied such a test. In the present paper it is applied to compare their formulae with the original equations of Möller *et al*, with data from both normal and nephritic subjects. The theoretically derived equation of Dole (5), which was apparently overlooked by both Williams and Bing, is also included in the comparison, and tentative conclusions are drawn concerning permeability changes in the renal tubules in chronic nephritis.

CLEARANCE FORMULAE

Maximum and standard clearance formulae of Austin et al (6), and Möller et al (3). Simultaneous observations of urea excretion rates, urine volumes, and blood urea concentrations made by Austin *et al* (6) and by Möller, *et al* (3) showed that the urea clearance, defined as the volume of blood containing the amount of urea excreted in 1 minute, was but little affected by urine flow changes in normal human subjects when the flow (per 1.73 sq. m. body area) exceeded an "augmentation limit" which was usually about 2 cc. per minute, but that when the urine flow fell below this limit the urea clearance fell with the urine flow, the clearance then becoming proportional approximately to the square root of the flow. Chesley (7, 8) confirmed the square root rule for urine flows down to about 0.35 cc. per minute, but found that when extreme dehydration reduced urine flow below this rate, further reduction in flow was accompanied by a more rapid fall in urea clearance, which then fell in direct proportion to the urine flow, rather than to its square root.

The above empirically observed effects of urine volume on the urea clearance in the 3 respective urine flow ranges are expressed by Equations *1, 2* and *3*, in which C_r represents the clearance calculated as UV/B, for any urine volume flow V (in cc. per minute), and U and B indicate the concentrations of urea in urine and blood respectively.

When V exceeds the augmentation limit of about 2:

1. $$C_r = C_m = \text{constant for each subject.}$$

C_m is the "maximum clearance" of Möller *et al* (3), and averages 75 for normal adults.[1]

When V is between the augmentation limit and 0.5 cc. per minute:

2. $$C_r = C_1 \sqrt{V}.$$

C_1, the clearance when $V = 1$, was called the "standard clearance" by Möller *et al* (3), who found that it averaged 54 for normal adults.

Chesley's formula for maximal urine concentration. When V is less than 0.35 cc. per minute the value of C_r approximates that expressed by Chesley's (7, 8) formula:

3. $$C_r = R_m \times V.$$

R_m is a constant, the maximum U/B ratio attainable by decreasing urine flow to its minimum. The average normal value of R_m is about 75. (In nephritis the value of R_m may fall to 3 or 4 (9) and be reached with urine volumes above 1 cc. per minute [see Table IV].)

Dole's equation. Dole (5) has derived an equation, based on estimation of tubular urea reabsorption in accordance with Fick's diffusion law, which covers the effects of all ranges of urine flow.[2] Dole's equation is:

4. $$C_r = C_m/e^{b/V} = C_m/\text{antilog}_{10}\frac{b'}{V}.$$

---

[1] Correction for body size in any clearance equation is made by using as $V_{corrected}$, the observed V multiplied by the factor, 1.73/(sq. meters body area), in the calculation of C_r as UV/B, and wherever V appears elsewhere in the equation. It has been shown (10) that clearances vary in direct proportion to body area in human subjects above the age of 1 year, and that the correction can be made by applying the factor 1.73/m². to V, 1.73 being taken as the average adult surface area.

[2] Dole expressed his equation as $C_r = F \times \phi \times e^{-k/V}$, where F is the amount of urea filtered per minute, ϕ is the fraction (about 0.60) that escapes reabsorption in the first fraction of the tubular segments, and $e^{-k/V}$ is the part of that fraction that escapes reabsorption in the final tubule and achieves excretion as urine. In terms of the maximal clearance, $C_m = F \times \phi$, and C_r approaches C_m when V becomes so large that $e^{-k/V}$ approaches unity. In Equation *4* we have written C_m in place of $F \times \phi$, and have used b to indicate specifically the constant k in the

C_m, as in Equation *1*, signifies the maximal clearance obtained with large urine volume flow. Dole's equation is based on the assumptions: (*1*) that a constant fraction (normally about 40 per cent) of the urea in the glomerular filtrate diffuses back into the blood from the lumina of the proximal tubules with the "obligatively" reabsorbed water (which is estimated to be about 90 per cent of that filtered); (*2*) that in a second section of the tubule further reabsorption of water, with negligible reabsorption of urea, brings the filtrate to its final volume (the urine volume, V); (*3*) that during passage of a third tubular segment a second fraction of the filtered urea is reabsorbed, without water, into the blood by passive diffusion, this fraction being calculable by Fick's diffusion law from the permeability of the tubular wall of this segment for urea and the final volume of the filtrate. C_m represents the cc. of blood that would be cleared of urea per minute if, of the total urea filtered in the glomeruli the approximately 60 per cent that escapes reabsorption in the proximal tubules were all excreted, while the fraction $1/e^{b/V}$ is the fraction of the 60 per cent that finally attains excretion after part has been reabsorbed in the third segment. The constant, b, is the product of the surface area of the walls of the third segments and their permeability for urea, which Dole estimates to be of the order of 0.001 of the permeability of erythrocytes for urea. The constant b' in Equation *4* is b multiplied by 0.4343 to change from exponent of e to exponent of 10. If b has a high value in a given subject the effect of urine volume change on clearance is high, and vice versa.

We have estimated the approximate mean normal values of C_m and b' of Equation *4* as 80 and 0.17, respectively, from the data on the 6 normal subjects in Table II of Möller *et al* (3). The value, 80, calculated for mean normal C_m of Equation *4*, is higher than the C_m value of 75, estimated by Equation *1* as the mean of clearances observed with urine volumes above an "augmentation limit" of about 2 cc. per minute. The Dole equation assumes that the clearance increases asymptotically to a maximum reached at higher urine output; hence this calculated maximum is greater than the mean observed in the V range above 2 cc.

As Dole points out, individual and pathological variations in b may be expected. However, application of Dole's equation to the normal data of Möller *et al* (3) and to their data from patients with Bright's disease indicates that in both normal and nephritic human subjects one can calculate for each subject fairly constant results for C_m from observed C_v and V values by assuming a value of 0.17 for b' in all the subjects.

factor $e^{-k/V}$. The equation $C_v = F \times \phi \times e^{-k/V}$, with ϕ assumed to be constant, is a simplified form of a more complete equation developed by Dole in which the value of ϕ is expressed as a function of the volume of filtrate escaping "obligative" reabsorption in the first segment. The mean normal value of ϕ is estimated as the ratio, (urea clearance with maximal urine flow):(inulin clearance), which is about 0.60 in normal men. It is usually about the same in nephritis, but may be nearer unity (11).

Williams' formula. Williams (2), from the data of Möller *et al* (3) on normal subjects, evolved the equation: $(U/B)^{1.103}V = K$. This can be transformed into:

$$5. \qquad C_v = C_1 V^{0.093},$$

where C_1, as in Equation *2*, indicates the value of C_v when $V = 1$, C_1 being 54 for the average normal subject.

Bing's formula. Bing (1) believes that the most accurate calculation of C_m can be made by estimating tubular reabsorption of urea, not from V, but from the ratio, U/B, as indicated by Equation *6*, the factor f being a function of U/B that Bing estimated from the data of Möller *et al* (3) on normal subjects. For plotting the curve of f against U/B, Bing gives the following pairs of simultaneous values for U/B and f, respectively: U/B 1 to 20, $f = 1$; 30, 1.15; 40, 1.30; 50, 1.43; 60, 1.54; 70, 1.60; 80, 1.64. With values of f varying from 1 to 1.64 according to U/B, Bing's formula is:

$$6. \qquad C_v = C_m/f.$$

Although Bing introduced his formula to replace V by U/B as a factor determining C_v, it can be shown that Bing's f is in fact a function of V. By plotting the logarithms of U/B against the logarithms of the corresponding f values given by Bing, a straight line is obtained, the equation of which is $f = \left(\frac{U}{20\,B}\right)^{0.37} = 0.33\ (U/B)^{0.37}$, and which gives Bing's f values within ±2 per cent, for values of U/B over 20 (f being 1 when $U/B < 20$). Substituting $0.33\ (U/B)^{0.37}$ for f in Equation *6*, and solving for $C_v = \frac{UV}{B}$ gives, as an expression of Bing's formula applicable when U/B exceeds 20:

$$7. \qquad C_v = 2.24\ C_m^{0.73} V^{0.27}.$$

When C_m has the average normal value of 75, the equation becomes:

$$8. \qquad C_v = 52.4\ V^{0.27}.$$

The value, 52.4, for $V = 1$, approximately agrees with the normal C_1 of 54 found by Möller *et al* (3).

Equation *8* is used in Figure 1 to include Bing's formula in the comparison of the effects of V change calculated by the different formulae for a subject of average normal clearance.

COMPARISON OF RESULTS BY DIFFERENT FORMULAE

Data used. Möller and McIntosh provide data on 6 normal subjects (3), for each of whom 12 to 20 clearances were determined with urine volumes varying over the widest ranges obtainable by varying the fluid intake, and for 6 nephritics with varying degrees of abnormally lowered urea clearance, for each of whom from 9 to 26 clearances were determined with similarly varied urine volume flows. These data are used to compute Tables I to IV. As an

additional normal subject, H. A. from a previous paper (6) is added: H. A. provides 20 clearances with V values well distributed over a wide range.

Procedure for comparison. To compare the relative accuracies with which the different formulae estimate the effect of V or U/B on C_v, values of the maximum clearance, C_m, obtainable by high urine flow for each subject, are calculated by the different formulae from the C_v values obtained with the various observed flows. For each individual the standard percentage deviation is computed of the C_m values calculated by each formula from the mean C_m of the subject calculated by that formula. It is assumed that the most accurate formula will be the one that yields the smallest average percentage deviation from the means.

By any of the formulae except that of Bing, one could calculate C_1 (clearance when $V = 1$) or the clearance at any other V, instead of C_m. Bing's formula (Equation *6*), however, applied as given by its author, serves only for calculation of the maximum clearance as a function of U/B.

The self-evident rearrangements of Equations *1*, *4* and *6* to calculate C_m are indicated in the column headings of Tables I and II.

For calculation of C_m by the square root relation expressed in Equation *2*, the relation is expressed as

$$9. \qquad C_m = C_v \sqrt{\frac{A}{V}},$$

where A is the augmentation limit of V, and is assumed to be a constant. A is taken as the V value at which both Equation *1* and Equation *2* indicate the same C_v value. If the mean normal values of C_m and C_1 *viz.* 75 and 54, are inserted in Equations *1* and *2*, respectively, both equations give the same value for C_v when $V = 1.93$. We have therefore used 1.93 as a constant value for A. Substituting 1.93 for A in Equation *9* gives:

$$10. \qquad C_m = C_v \sqrt{\frac{1.93}{V}} = 1.39\ C_v/\sqrt{V}.$$

Equation *10* is used to compute values for the clearances by the formula of Möller, McIntosh and Van Slyke (3) in Tables I to IV for urine volumes below 1.93 cc. per minute. The assumption of 1.93 as a constant augmentation limit for all the subjects, normal and nephritic, introduces a variable error, since, as shown in the original papers (3, 4), the augmentation limit varies somewhat from subject to subject. The results in Tables I to IV indicate, however, that the error from assumption of a constant value for A is not great in either normal or nephritic subjects. The possible explanation for the apparent fact that the reduction of renal function in nephritis may not greatly affect the augmentation limit will be discussed later.

To estimate C_m by Williams' formula (Equation *5*) we have calculated, from observed C_v values, the clearance for a urine flow of 20 cc. per minute. As shown by Figure 1, Williams' formula for the average normal subject indicates a clearance, when V approaches 20, that approximates the 75 cc. value found by direct observation (3) as the average normal C_m. Also a urine flow of 20 cc. per minute is about the maximum obtainable by water diuresis.

Letting C_{20} indicate the clearance obtained when $V = 20$, we have from Equation *5*, according to Williams:

$$C_{20}:C_v = (20/V)^{0.093} = 1.32\,/\,V^{0.093},$$

whence:

$$11. \qquad C_{20} = 1.32 C_v/V^{0.093}.$$

The C_{20} calculated by Equation *11* is given in tables as the C_m calculated by Williams' formula.

RESULTS OF COMPARISONS

The means and standard deviations of the series of normal subjects of Möller, McIntosh, and Van Slyke (3) are given in Table I, those of their nephritic subjects (4) in Table II. Tables III and IV give the complete data on normal subject H. A., and on the nephritic, Gia, who showed the lowest clearance values of the group in Table II.

The relative accuracies of the different formulae applied to 3 normal subjects are illustrated by Figure 1. The curves are calculated for the hypothetical average normal adult; they represent C_v values calculated by the indicated equations with mean normal values for their constants. Subject H. A. is a normal man whose constants closely approximate these values. The points for very low urine volumes are taken

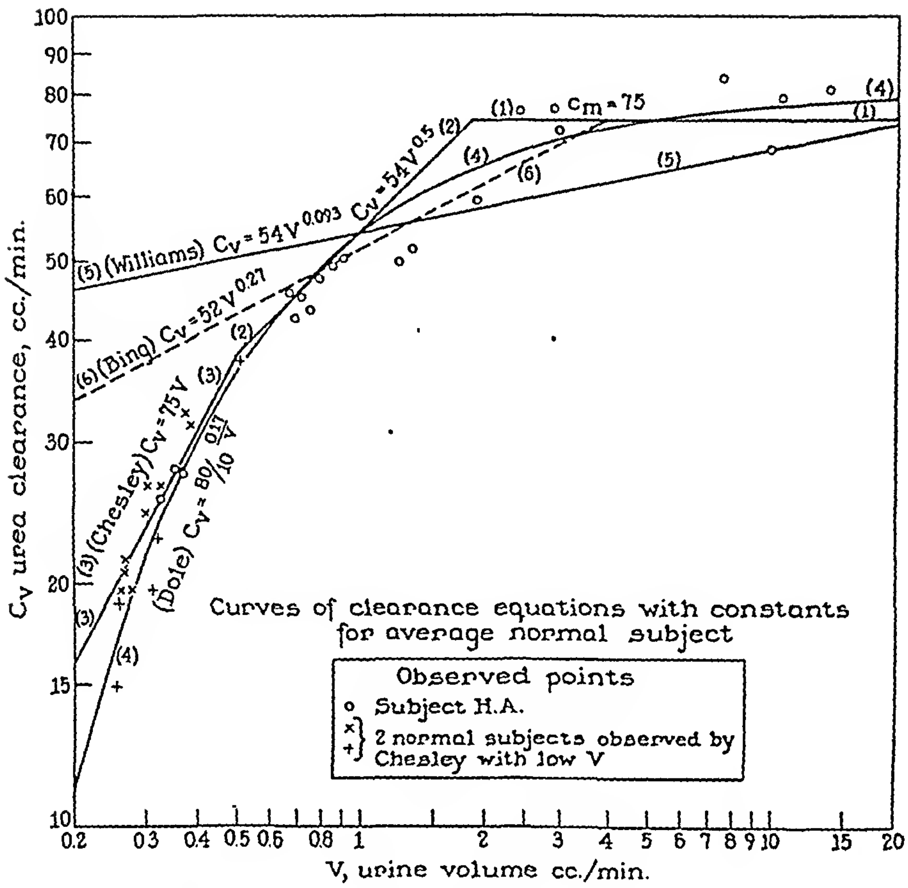

Fig. 1. Comparison of Observed Relation of Urea Clearance and Urine Volume with Relations Calculated by the Indicated Formulae for the Average Normal Subject. The number in parentheses on each curve indicates the number of the equation, in the text, by which the curve is calculated.

from data of Chesley (8) on 2 subjects who gave R_m values approximating 75. The curves illustrate the facts that Dole's theoretical equation appears to apply adequately to all urine volumes, that the 3 empirical equations of Möller *et al* (3) and Chesley (7, 8) represented by the broken-line curve, 1–2–3, fit approximately within their respective ranges of urine flow, and that the Bing and Williams formulae are less accurate, especially for low urine flows.

From Tables I and II it is evident that C_m values calculated by the formulae of Bing and Williams show consistently for each subject, both normal and nephritic, greater standard deviations from the mean of the subject than do C_m values calculated by the original "maximum" and "standard" clearance formulae of Möller, McIntosh and Van Slyke (3), or by the equation of Dole (5).

Tables III and IV show how the errors of both the formula of Bing and that of Williams are particularly great when these formulae are applied to clearances observed with low urine flows, where error in relating maximal water reabsorption to urea reabsorption in the tubules becomes most apparent.

That the standard deviations of individual C_m values in Tables I and II are not significantly greater for C_m calculated by the formulae of Möller *et al* than by Dole's equation, is attributable to the fact that but few of the urine flows in the observations recorded were below 0.5 cc. per minute. The results confirm Dole's conclusion with regard to clinical use of clearance formulae, that "the 'maximal' and 'standard' urea clearance formulae are adequate for their purposes, except for conditions of unusually small urine flows."

The conclusions concerning the relative accuracy of the "maximum" and "standard" clearance formulae compared with Williams' exponential formula reached by the above analysis

TABLE I

Normal subjects. Variability of maximal urea clearance, C_m, calculated from observed clearance, C_r, by different formulae *

Figures in parentheses indicate percentage of average normal C_m.†

Subject and no. of observations	Range of urine volume V	Formulae: M. M. and V. S. (3) $C_m = C$ when $V > 1.93$; $C_m = 1.39\, C_r/\sqrt{V}$ when $V < 1.93$		Bing (1) $C_m = C_r$ when $U/B < 20$; $C_m = fC_r$ when $U/B > 20$		Williams (2) $C_{22} = 1.32\, C_r/V^{0.073}$		Dole (5) $C_m = C_r \times \text{antilog}\, \frac{0.17}{V}$	
		Mean C_m	St. dev. from mean	Mean C_m	St. dev. from mean	Mean C_m	St. dev. from mean	Mean C_m	St. dev. from mean
	cc. per minute		*per cent*	*cc. per minute*	*per cent*	*cc. per minute*	*per cent*	*cc. per minute*	*per cent*
L. L. 12	0.47 to 12.33	79 (105)	±14.5	83 (111)	±14.3	79 (111)	±21.1	86 (108)	±12.2
J. F. M. 12	0.60 to 8.58	68 (91)	±12.7	70 (93)	±17.4	71 (100)	±19.3	75 (94)	±12.0
A. H. 12	0.57 to 10.83	64 (85)	±10.3	66 (88)	±22.0	67 (94)	±22.9	71 (89)	±14.1
W. N. 13	0.37 to 12.50	71 (95)	±13.6	73 (97)	±24.1	70 (99)	±22.1	78 (97)	±14.1
V. S. 14	0.50 to 16.25	83 (111)	±11.3	86 (115)	±14.6	82 (115)	±19.9	90 (113)	±11.8
J. C. B. 20	0.80 to 12.47	61 (81)	±10.2	59 (79)	±14.6	65 (92)	±16.4	65 (81)	±11.6
H. A. 20	0.32 to 13.40	72 (96)	± 9.2	71 (95)	±19.4	69 (97)	±24.9	80 (100)	± 7.2
Mean		71 (95)	±11.8	72 (96)	±16.5	72 (101)	±21.0	79 (99)	±11.8

* Figures for first 6 subjects include all the data on Table II of Möller, McIntosh, and Van Slyke (3), except one obviously erroneous clearance of W. N. Data on H. A. are from Austin, Stillman, and Van Slyke (6).

† Average normal values assumed are 75 for C_m by the equations of M. M. and V. S. and Bing, 71 for C_{22} calculated from C_1 of 54 by Williams' equation, 80 for C_m by Dole's.

are contrary to the conclusion of Williams based on the same data, in so far as the normal subjects are concerned, which were the only group considered by Williams. The difference appears to be due to different statistical procedures used by Williams and by ourselves in estimating the accuracy of the respective formulae. Williams calculated V from observed values of U/B both by his formula and by the "maximum" and "standard" clearance formulae (Equations *1* and *2*), using for all subjects average normal values of the constants of the respective equations. He calculated as d for each equation the mean difference between the V values thus calculated and the observed V values. His formula gave the lower mean d, and he therefore concluded that it was the more accurate. In these calculations Williams used for all subjects as constants the values of C_1 or C_m, *viz.* 54 and 75, found by Möller *et al* (3) as the *average* for normal adults. Thus the equations used (from Equations *5*, *1* and *2*, respectively) to calculate V were: $V = \left(54\,\frac{B}{U}\right)^{1.1[illegible]}$ (Williams [2]), $V = \frac{75\,B}{U}$ (Möller *et al* for V over 1.93) and $V = \left(54\,\frac{U}{B}\right)^2$ (Möller *et al*) for $V < 1.93$. The use of the same C_m and C_1 values for every subject of the group introduces errors into the V values calculated when the actual C_m and C_1 of any subject deviate from 75 and 54, and are

TABLE II

Nephritic patients. Variability of maximal urea clearance, C_m, calculated from observed clearance, C_v, by different formulae. Data of Möller, McIntosh and Van Slyke (4)

Figures in parentheses indicate percentages of average normal.*

Subject	Range of urine volumes V†	Formulae										
		M. M. and V. S. (3) $C_m = C_v$ when $V > 1.93$; $C_m = 1.39\ C_v/\sqrt{V}$ when $V < 1.93$		Bing (1) $C_m = C_v$ when $U/B < 20$; $C_m = fC_v$ when $U/B > 20$		Williams (2) $C_m = 1.32\ C_v/V^{0.033}$		Dole (5) $C_m = C_v \times \text{antilog}\ \frac{b'}{V}$				
								With $b' = 0.17$ for all subjects		With estimated best b' for each subject		
		Mean C_m	St. dev. from mean C_m	Mean C_m	St. dev. from mean C_m	Mean C_m	St. dev. from mean C_m	Mean C_m	St. dev. from mean C_m	b'	Mean C_m	St. dev. from mean C_m
	cc. per minute	*cc. per minute*	*per cent*	*cc. per minute*	*per cent*	*cc. per minute*	*per cent*	*cc. per minute*	*per cent*		*cc. per minute*	*per cent*
Chi. 9	0.28 to 9.27	85 (113)	±12.3	78 (104)	±23.8	74 (104)	±26.0	97 (121)	±13.0	0.13	85	± 9.3
Jac. 24	0.32 to 6.37	31.3 (39)	± 8.0	24.8 (33)	±25.7	29.8 (42)	±24.4	34.3 (43)	± 8.5	0.19	36.0	± 8.4
Cic. 18	0.39 to 5.07	24.8 (31)	±18.9	19.3 (26)	±32.2	23.2 (33)	±29.4	27.3 (34)	±18.9	0.21	30.2	±14.3
Val. 26	0.66 to 5.99	17.4 (22)	±12.1	13.3 (18)	±13.0	16.7 (23)	± 8.5	18.7 (23)	± 9.6	0.09	15.6	± 7.1
Wol.‡ 20	0.81 to 3.55	11.7 (14.6)	±17.0	11.2 (14.9)	±21.9	13.7 (19.3)	±19.3	13.6 (17)	±15.2	0.33	16.3	±14.5
Gia. 14	0.71 to 3.96	11.0 (14.7)	±10.9	8.7 (11.6)	±22.3	11.1 (15.6)	±18.2	12.1 (15.1)	±11.1	0.24	14.1	± 9.5
Mean standard deviation			±12.7		±23.1		±21.0		±11.6	0.20		±10.5

* See footnote † of Table I.

† Values of V are corrected for body size by multiplying observed V by the factor 1.73/sq. meter surface area (10).

‡ One clearance of subject Wol. is omitted from the calculations because of obvious error in the data.

particularly large when the square root formula is applied, because with this formula the error of the calculated V increases as the error of the square of the assumed C_1. In the case of normal subject J. C. B. (3), with a C_1 value of 41, the use of 54 yields calculated V values with a plus error of almost 100 per cent. Such errors could be cancelled only by the use of a large series of subjects. The statistical procedure of Williams compares the results obtained by adding errors from 2 sources: (*1*) the error of each formula in expressing the relation of V to C_v, and (*2*) the error caused by using the group constant for each subject. The difference between his results and ours appears attributable to predominance of errors from the second source in his calculated V values.

Significance of non-decrease of the augmentation limit and the constant, b, *of Dole's equation in nephritic subjects as indication of increased tubular permeability to urea*

It was a matter of surprise to Möller *et al* (4) to find that in patients with glomerular nephritis and markedly reduced clearances the augmentation limit was usually of the same order of magnitude as in normal subjects. If the disease process affected renal function by inactivation of part of the nephrons, corresponding to the destroyed glomeruli seen histologically, while the remaining nephrons functioned normally, one might expect that the augmentation limit would fall parallel with the number of functioning nephrons, and hence with the clearance.

TABLE III

Maximal urea clearance of a normal subject calculated from observed clearances with different urine volume flows (Subject H. A. [6])

Urine volume V	Observed clearance $C_r = \frac{UV}{B}$	Maximal clearance, C_m, calculated by different formulae from observed clearances: Equations of M. M. and V. S. (3)	Formula of Bing (1)	Equation of Williams (2)	Equation of Dole (5) with $b' = 0.17$
cc. per minute		*cc. per minute*	*cc. per minute*	*cc. per minute*	*cc. per minute*
0.326	25.5	62	42	37	84
.355	26.3	65	45	40	79
.368	27.6	64	45	40	80
.674	45.9	78	74	63	83
.694	42.5	71	66	58	75
.722	45.1	74	70	61	78
.756	43.5	70	66	58	73
.800	47.8	74	72	64	78
.826	47.4	72	73	64	76
.917	50.5	73	75	67	78
1.26	60.0	74	85	77	82
1.35	51.8	62	66	66	69
1.925	59.6	60	69	74	73
2.46	76.2	76	89	92	90
2.95	77.0	77	84	92	88
2.99	72.5	72	87	86	83
7.50	84.0	84	84	93	88
9.64	68.8	69	69	74	72
10.40	79.5	79	79	84	83
13.40	81.4	81	81	84	84
Mean:					
cc. per minute		72	71	69	80
Per cent of average normal		96	95	97	100
Standard deviation from mean:					
cc. per minute		±6.6	±13.8	±17.2	±5.8
per cent of mean		±9.2	±19.4	±24.9	±7.2

From the same conception one would expect the constant, b, of Dole's equation to fall parallel with the clearance. This constant is defined by Dole as the product, $b = a \times h$, where a represents the total area of the walls of the distal segments of the functioning tubules and h represents the permeability of the walls of the distal segments for urea. Destruction of part of the nephrons, and consequent decrease in the area, a, would result in a proportional decrease in b, unless the permeability, h, were increased in the tubules that remained functioning. It appears probable that such a permeability increase occurs, and provides an explanation of 2 aspects of renal function in advanced nephritis, viz. (*1*) the maintenance of the augmentation limit near the normal 2 cc. of urine per minute, and of the b constant at normal, or even higher than normal levels, in many cases, and (*2*) the inability of the damaged kidneys to excrete urine with high urea concentration.

We have estimated values of b' of Equation *4* for the 6 nephritic patients of Table II by plotting smoothed curves of C_r against V, and using values of C_r and V from points on the upper and lower parts of each curve, respectively, to calculate by simultaneous equations the constants, C_m and b' of Equation *4*. The results are given in the last 3 columns of Table II. Only in case Wol. is the b' value markedly lower than in normal subjects, and the average, 0.20, is higher than the value 0.17 found as the mean for the normal subjects in Table I. In patient Val., despite the fact that the nephritis was so far advanced that the urea clearance was only $\frac{1}{5}$ of normal, the augmentation limit was so high that urine volumes up to 3.5 cc. per minute failed to locate the limit, and the b' value, 0.33, was twice the normal. One might deduce that the permeability of the functioning tubules for urea was 10-fold normal.

The apparent reason for such behavior is that these tubular segments (presumably the distal),

TABLE IV

Maximal urea clearances of nephritic patient Gia. calculated from observed clearances with different urine volume flows

Urine volume V	Observed clearance $C_r = \frac{UV}{B}$	$\frac{U}{B}$	Maximal clearance, C_m, calculated by different formulae from observed clearances: Equation of M. M. and V. S. (3)	Formula of Bing (1)	Equation of Williams (2)	Equation of Dole (5) With $b' = 0.17$	Equation of Dole (5) With $b' = 0.24$
cc. per minute		*ratio*	*cc. per minute*	*cc. per minute*	*cc. per minute*	*cc. per minute*	*cc. per minute*
0.71	5.58	8.0	9.2	Same as C_r	7.6	9.7	12.2
0.76	6.76	8.9	10.8	Same as C_r	9.1	11.3	14.0
0.85	7.62	9.0	11.3	Same as C_r	10.2	12.1	14.6
0.88	7.04	8.6	10.4	Same as C_r	9.4	11.0	13.2
0.92	8.01	8.7	11.6	Same as C_r	10.7	12.3	14.6
0.92	8.46	9.2	12.3	Same as C_r	11.2	12.9	14.6
0.97	7.50	7.7	10.6	Same as C_r	9.9	11.2	13.2
0.99	9.07	9.1	11.7	Same as C_r	12.0	13.5	14.8
1.11	9.70	8.7	12.8	Same as C_r	12.7	13.5	15.9
2.17	10.11	4.8	10.1	Same as C_r	12.4	12.2	13.0
3.20	12.20	3.8	12.2	Same as C_r	14.4	13.8	14.5
3.96	12.00	3.1	12.0	Same as C_r	13.9	13.3	13.8
Mean:							
cc. per minute			11.0	8.7	11.1	12.1	14.1
per cent of average normal			14.7	11.5	15.6	15.1	17.6
Standard deviation from mean:							
cc. per minute			±1.20	±1.94	±2.02	±1.38	±1.34
per cent of mean			±10.9	±22.3	±18.2	±11.1	±9.5

where urea reabsorption is variable in the still functioning nephrons, are damaged in such a manner that they have an increased permeability to urea, so that urea concentration in the urine issuing from them must be kept at a lower level than in the normal tubule in order to prevent reabsorption of urea from becoming significant. In order to keep the urea concentration at the necessary lower level the volume of urine issuing per minute from each tubule must be kept greater than in the normal tubule. The permeability of the tubules approaches that observed by Richards (12) in the kidneys of frogs poisoned with mercuric chloride, in which the permeability was so increased that, although glomerular filtration appeared even more active than normal, the filtrate was completely reabsorbed in the tubules, with resultant anuria.

By maintaining a sufficiently large urine volume the nephritic kidney can apparently prevent the reabsorption of urea from exceeding the 40 per cent observed in the normal human kidney when urine flows exceed about 2 cc. per minute, but in order to prevent greater reabsorption the nephritic kidney must maintain a higher *ratio* of urine flow to glomerular filtrate than the normal kidney.

As a consequence of this condition, the $U:B$ ratio of urea concentrations at the augmentation limit does not remain, in the nephritic kidney, at the normal level 20:1 as assumed by Bing (1), but falls progressively as the functioning tubules become more damaged, *e.g.*, in the case in Table IV the $U:B$ appears to be in the neighborhood of 3 or 4 when urine flow nears the augmentation limit.

Significance of urine volume in maintaining elimination by the nephritic kidney

Lashmet and Newburgh (13) and Marriott (14) have emphasized that in nephritis maintenance of a large flow of urine is necessary when ability to concentrate is decreased. Figure 2 indicates how a study of the urea clearance with different urine volumes in the individual patient can indicate the volume output that is needed to approximate maximal efficiency of urea excretion. Cases Val. and Wol. both had about the same urea clearance, 20 per cent of normal when their urine volumes were as high as 4 cc. per minute. When the urine flows of the 2 patients were diminished, however, they showed markedly different effects. Val. maintained his clearance at approximately the same level until the urine output fell to about 1.2 cc. per minute, while in the case of Wol., fall of urine volume to 1.2 cc. per minute was accompanied by a drop of 40 per cent in the urea clearance below its value when urine flow was 3 or 4 cc. It appears that in Val. a daily excretion of 1.7 liters per 24 hours (1.2 cc. per minute) sufficed to obtain nearly

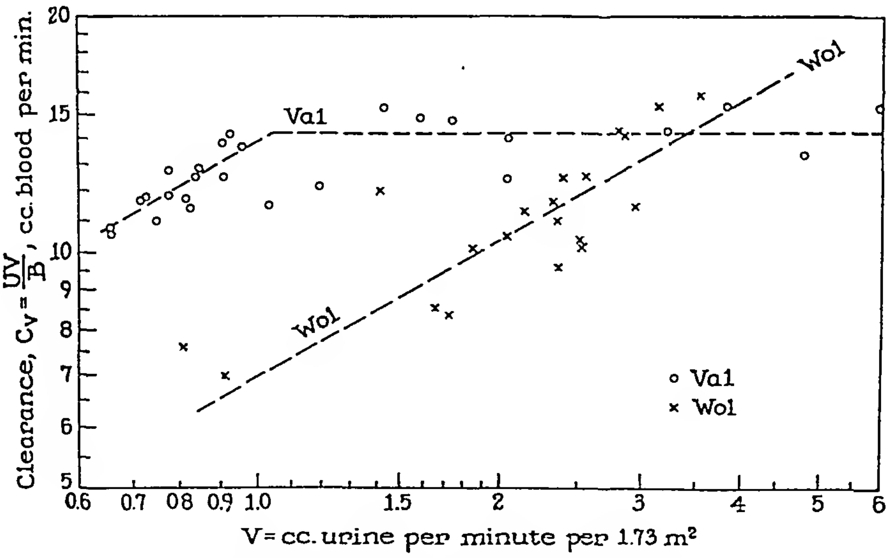

FIG. 2. DIFFERENT EFFECTS OF URINE VOLUME FLOW ON UREA CLEARANCE IN 2 NEPHRITIC PATIENTS

maximal efficiency of urea excretion, while in Wol. 3 times as great a volume was needed.

SUMMARY

The "maximum" and "standard" urea clearance formulae of Möller, McIntosh, and Van Slyke have been compared with formulae proposed by Bing, by Williams, and by Dole for estimating the effect of urine volume on urea clearance. The accuracy of the respective formulae has been estimated by comparing the constancy with which the maximal clearance, obtainable with high urine volume, could be calculated from clearances observed with lower urine volumes by the respective formulae for each of a number of normal and nephritic subjects.

The formulae of Bing and of Williams proved to be less accurate than those of Möller *et al* and of Dole.

The conceptions of Dole's theoretically derived formula have been used to explain, from increased permeability of damaged renal tubules, the different effects of urine volume on urea excretion in different nephritic subjects, and the loss in nephritis of ability to excrete urine of high urea concentration.

Examples are given indicating that, by observing the effect of urine volume on urea clearance in nephritic patients, it may be possible to estimate the urine volume flow required for optimal urea excretion.

BIBLIOGRAPHY

1. Bing, J., The calculation of urea clearance with varying degrees of urine concentration. Acta Med. Scand., 1946, 126, 199.
2. Williams, E. M. V., Urea excretion by rabbits. J. Physiol., 1946, 105, 223.
3. Möller, E., McIntosh, J. F., and Van Slyke, D. D., Studies of urea excretion; relationship between urine volume and rate of urea excretion by normal adults. J. Clin. Invest., 1928, 6, 427.
4. Möller, E., McIntosh, J. F., and Van Slyke, D. D., Studies of urea excretion; relationship between urine volume and rate of urea excretion by patients with Bright's disease. J. Clin. Invest., 1928, 6, 485.
5. Dole, V. P., Back diffusion of urea in the mammalian kidney. Am. J. Physiol., 1943, 139, 504.
6. Austin, J. H., Stillman, E., and Van Slyke, D. D., Factors governing the excretion rate of urea. J. Biol. Chem., 1921, 46, 91.
7. Chesley, L. C., The validity of the calculation of standard urea clearances from low urine volumes. J. Clin. Invest., 1937, 16, 652.
8. Chesley, L. C., Urea excretion at low urine volume. The calculation of minimal urea clearance. J. Clin. Invest., 1938, 17, 119.
9. Van Slyke, D. D., McIntosh, J. F., Möller, E., Hannon, R. R., and Johnston, C., Studies of urea excretion; comparison of the blood urea clearance with certain other measures of renal function. J. Clin. Invest., 1930, 8, 357.
10. McIntosh, J. F., Möller, E., and Van Slyke, D. D., Studies of urea excretion. III. The influence of body size on urea output. J. Clin. Invest., 1928, 6, 467.
11. Chasis, H., and Smith, H., The excretion of urea in normal men and in subjects with glomerulonephritis. J. Clin. Invest., 1938, 17, 347.
12. Richards, A. N., Direct observations of changes in function of the renal tubule caused by certain poisons. Tr. A. Am. Physicians, 1927, 44, 64.
13. Lashmet, F. H., and Newburgh, L. H., A comparative study of the excretion of water and solids by normal and abnormal kidneys. J. Clin. Invest., 1932, 11, 1003.
14. Marriott, H. L., Water and salt depletion. Brit. Med. J., 1947, pp. 245, 285 and 328.

THE INFLUENCE OF ORALLY ADMINISTERED ALKALI AND ACID ON THE RENAL EXCRETION OF QUINACRINE, CHLOROQUINE AND SANTOQUINE[1]

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Haag and Larson (1) demonstrated in 1942 that in the case of nicotine the extent of urinary excretion of a chemical may be related to the reaction of urine. They emphasized the importance of taking into consideration the dissociation constant of a drug and the relative reabsorbability of the free and dissociated base. Extending these studies to the urinary excretion of quinine in man, Haag, Larson and Schwartz (2) found in 1943 that the urinary output could be doubled by passing from an alkaline to an acid urine and they ascribed the difference to greater resorption of quinine from the urinary tract when the urine is alkaline.

In connection with studying the behavior of newer antimalarial drugs in man, the extent of the influence of urinary acid-base balance upon renal excretion was re-examined. Parallel studies by Emerson and Dole (3) indicated that renal clearance of quinacrine is subject to 100-fold variations due principally to 2 variables, the urinary pH and the renal plasma flow. Work conducted at the same time by the Army Malaria Research Unit at Oxford (4) emphasized the striking parallelism between urinary excretion of quinacrine and ammonia. They found that the ratio of urinary quinacrine to urinary ammonia is directly proportional to the plasma quinacrine concentration. Trager and Hutchinson (5) in 1946 similarly found the urinary excretion of quinacrine to be closely correlated with the urinary excretion of ammonia.

The present investigation was designed to ascertain the effect of oral ingestion of alkali and acid upon the renal excretion of this general type of organic compound. The substances selected for study were quinacrine, chloroquine (SN-7618), and santoquine (SN-6911).

METHODS AND MATERIALS

The patients utilized in this study were from the middle and older age groups. No restriction was placed on the diet or upon water intake during the observations and the patients were permitted the freedom of the ward. All drugs were administered orally and according to a single general dosage pattern. A high initial dose was administered during the first 24 hours and this was followed by the serial administration of smaller maintenance doses. Several days were allowed to elapse for the stabilization of the plasma drug concentration before observations were begun.

Alkali was administered orally in the form of sodium bicarbonate at a total daily dose of 20 grams. This was given in doses of 2.5 grams at 2-hour intervals during the day for 7 doses and once during the night. Acid was administered orally in the form of ammonium chloride at a total daily dose of 8 grams. The dosage schedule was the same as for the bicarbonate, each dose being 1.0 gram.

Plasma and urine estimations were performed by methods developed by Brodie. Quinacrine was determined fluorometrically by the single extraction procedure using ethylene dichloride (6); the alkaline wash was used in the urine determinations but not in the plasma determinations. The ethylene dichloride extract was made acid with trichloracetic acid (Masen [7]). Santoquine was estimated by the colorimetric procedure depending upon a salt formation with methyl orange (Brodie *et al* [8]).

The recently developed specific method (1947) for the analysis of chloroquine (9), involving the use of heptane and alkaline washes, was not yet available at the time of the present study. The procedure used involved the extraction of chloroquine with ethylene dichloride at pH 11, followed by the return of the drug to dilute acid. The acid solution was buffered at pH 9.5 and subjected to ultra-violet irradiation for a 1-hour period. Concentration was estimated by measurement of fluorescence generated by the irradiation. This procedure includes in the measurement interfering degradation products of chloroquine which are found in plasma to the extent of 20 to 50 per cent. However, it is quite specific for urine since only negligible amounts of these degradation products appear in urine.

[1] This work was carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and New York University.

Urine pH was determined in all cases on freshly voided urine samples using a glass electrode. No precautions were taken to maintain CO_2 tensions in the transfer of fluid. Consequently the data on urinary pH must be taken as close approximations rather than precise estimations of the pH of bladder urine. During the period of alkali administration the urinary pH rose to values between 7.5 and 8.0; during the acid period the pH fell to values of 4.0 to 5.0.

RESULTS

Quinacrine

The experimental data obtained on 4 patients receiving maintenance doses of 0.2 and 0.3 grams of quinacrine dihydrochloride are summarized in Table I. The amounts and concentrations noted are expressed in terms of free base. The control values for the renal excretion of quinacrine varied from 1 to 3 per cent of the daily dose. Excretion was reduced to 0.07 to 0.3 per cent of the daily dose during alkalinization and increased to 2 to 6 per cent during acidification. There was in individual cases a 14- to 25-fold increase in going from one state to the other. As is to be expected, such a change is not reflected in the plasma quinacrine concentration since the extent of renal excretion in relation to the oral dose is low in all cases. The plasma level of quinacrine remained roughly constant in 1 patient who was at equilibrium before the observations were begun, and increased as a more or less linear function of time in the 2 who had not as yet achieved an equilibrium plasma quinacrine concentration.

TABLE II

The influence of orally administered alkali and acid on the renal excretion of chloroquine (SN–7618)

Patient	Si.	Wo.	Ke.	Al.	Ch.
Drug intake	*200 mgm. per day*	*400 mgm. per day*	*400 mgm. per day*	*400 mgm. per day*	*400 mgm. per day*
	Urinary excretion	Urinary excretion	Urinary excretion	Urinary excretion	Urinary excretion
	milligrams per 24 hours				
Control period	113 104 75	30 105 99	133 84 119	62 110 103	110 60
$NaHCO_3$ *20 grams daily for 3 days*	47 24 41	55 26 17	53 34 37	136 79 61	60 27 30
NH_4Cl *8.0 grams daily for 3 days*	119 188 137	54 75 114	119 208 205	147 192 317	103 80

Chloroquine

The experimental data obtained on 5 patients receiving 0.2 or 0.4 grams of chloroquine daily are summarized in Table II. The daily excretion, in terms of percentage of the daily dose, was high in all patients as compared to quinacrine. This varied in the case of chloroquine, from 15 to 50

TABLE I

The influence of orally administered alkali and acid on the renal excretion of quinacrine

Patient	Wr.		Wo.		Ni.		Na.	
Drug intake	*300 mgm. per day*		*200 mgm. per day*		*200 mgm. per day*		*200 mgm. per day*	
	Urinary excretion	Plasma level	Urinary excretion	Plasma level	Urinary excretion	Plasma level	Urinary excretion	Plasma level
	mgm. per 24 hrs.	*µg. per L.*	*mgm. per 24 hrs.*	*µg. per L.*	*mgm. per 24 hrs.*	*µg. per L.*	*mgm. per 24 hrs.*	*µg. per L.*
Control period	2.92 7.47 5.98	81	4.63 3.7	54	1.93 0.80	23 31	7.14 4.38 9.3	50 54
$NaHCO_3$ *20 grams daily for 3 days*	1.32 0.9 0.98		1.73 0.66 0.54	40 59	0.33 0.27 0.13	48 55	2.1 0.79 0.42	87 97
NH_4Cl *8.0 grams daily for 3 days*	5.85 13.6 13.9		6.65 8.25	48	0.14 1.8 3.2	67	5.4 10.3 12.1	102

per cent in the control periods; was reduced to 7 to 20 per cent during alkalinization and was increased to 20 to 90 per cent during acidification.

Santoquine

Observations on santoquine were limited to a single individual. However, they were designed to stress the effects of changes in the renal excretion of drug upon the maintenance of the plasma santoquine concentration while on a constant drug intake. Each phase of alkali and acid administration was of 5 days' duration and 2 complete cycles of alkali-acid treatment were obtained (Table III).

TABLE III

The influence of orally administered alkali and acid on the renal excretion of santoquine (SN-6911)
Patient McK.
Drug intake: *400 mgm. per day*

	Urinary excretion	Plasma concentration
	mgm. per 24 hrs.	*micrograms per liter*
Control period	154	
	107	
	202	410
	190	400
$NaHCO_3$ *12.0 grams daily for 5 days*	36	
	77	
	56	
	82	630
NH_4Cl *6.0 grams daily for 5 days*	90	
	384	738
	290	685
	249	
	266	418
$NaHCO_3$ *20.0 grams daily for 5 days*	199	
	49	432
	37	
	60	775
	40	1010
NH_4Cl *8.0 grams daily for 5 days*	166	
	181	881
	191	
	149	343
	183	324

It should be noted that a large portion of the daily dose of santoquine is excreted in the urine. This was perhaps more in the patient utilized than is usual. During the control period average renal excretion accounted for 41 per cent of the daily dose. This was decreased to an average of 16 per cent during the first alkalinization and increased to an average of 64 per cent during the first acidification. Similar results were obtained during the second cycle.

As is to be expected in such a circumstance, the plasma santoquine concentrations reveal a cycle which can be correlated with the intervals of low and high renal excretion. During the alkaline phase of low drug excretion, the plasma level slowly rose; during the acid phase of high excretion, the plasma level fell.

DISCUSSION

It is apparent from the data that the renal excretion of the anti-malarial drugs is dependent upon a series of variables in addition to the concurrent plasma concentration. These variables are of sufficient importance in the determination of the renal drug excretion, that the latter may not be used directly as an approximation method to estimate the general level of the plasma drug concentration.

The refinement introduced by the British workers (4) involving the simultaneous measurement of urinary ammonia and quinacrine does permit of an indirect determination of plasma quinacrine from measurements on urine alone. It appears to us that ammonia excretion may be governed by urinary acid-base balance in a manner similar to the other weak organic bases, such as the anti-malarial drugs. The ammonia measurement provides an index of variation in urinary acid-base balance. By taking the quinacrine concentrations in relationship to ammonia excretion, the Oxford workers were able to eliminate the variable of pH. Trager and Hutchinson (5) seem to ascribe a causal role to ammonia in promotion of urinary excretion; it appears far more likely that ammonia and quinacrine excretions are concomitantly altered in a similar manner by urinary changes in hydrogen ion concentration.

The experimental data are interesting for yet another reason. It must be assumed that at least a portion of the drug eliminated by the kidneys is excreted by a process other than glomerular filtration. This follows from a consideration (in the case of quinacrine) of the extent to which it is bound to the non-diffusible constituents of plasma (some 80 to 90 per cent of the total) and the observed rates of excretion. The mechanism responsible for such tubular excretion as occurs ap-

pears to be quite different from those responsible for the tubular excretion of such substances as phenol red, creatinine, diodrast, etc. The latter manifest considerable stability whereas this does not obtain for the tubular excretion either of quinacrine or of chloroquine and santoquine.

Data are not available with which to define the mechanisms involved though there is sufficient to warrant certain reasonable speculations. It is suggested that a small portion of the excreted anti-malarial drug is filtered at the glomerulus, the amount determined by the glomerular filtration rate, and the concentration of unbound drug in plasma water. A second portion is considered to be added to this in the proximal segment by a process of active tubular excretion similar to the conventional processes considered above. The combined filtered and secreted drug is presented to the distal segment of the nephron in concentrated form due in part to the reabsorption of water in the proximal tubule and loop of Henle. In the distal segment, a process of adjustment of hydrogen ion concentration takes place followed by equilibration of drug across the tubular membrane. The data are in keeping with the assumption that free base is more freely diffusible across the cells of the distal segment than the monoacidic and diacidic salts of the drugs studied. Such being the case there may be expected to be net gain or loss of drug depending upon the relation between the hydrogen ion concentration of the tubular urine and that of peritubular fluid (*ca.* 7. 34). Alkalinization of the tubular urine would increase the proportion of drug in the form of free base and in this way would promote reabsorption; acidification would counteract reabsorption by reducing the proportion of free base. A somewhat similar mechanism has been suggested to cover the renal tubular excretion of neutral red in the amphibian kidney (10).

The observation of Trager and Hutchinson (5) that the plasma quinacrine level rose soon after oral ingestion of large doses of ammonium chloride, would seem to provide another basis for the altered urinary excretion. The elevation in plasma level observed in their experiment is of brief duration and is in keeping with the increased plasma levels observed in dog experiments (11) in which acute acidosis was produced by CO_2 administration. The plasma quinacrine measurement in the present study revealed no significant changes in level since by design the blood sampling and ammonium chloride administration were timed to elicit only sustained changes. The promotion of urinary excretion of quinacrine by ammonium chloride appears to us not to be secondary to plasma drug elevation but rather a direct result of the acid urine counteracting drug reabsorption in the tubules.

Relatively large changes in excretion rate are expected to be reflected in changes in the plasma drug level on a constant regimen of oral dosage only when the absolute rate of excretion of the substance is high. This phenomenon is brought out strikingly in the patient receiving santoquine. During the acid periods drug excretion amounted to as much as 50 to 75 per cent of the orally administered dosage. The effective dosage was thereby reduced to ½ or ¼ of the actual administered dose. During the alkaline period, the per cent of drug excretion was small, namely 20 per cent of the ingested dose. Under these circumstances, the effective oral dose was as high as 80 per cent of the actual administered dosage.

Quinacrine excretion, though markedly altered by acid-base changes, remains in all circumstances such a small percentage of the orally administered dosage that changes in renal elimination would not be reflected in the maintained plasma level. The changes in drug excretion, from 0.2 per cent during the alkaline period to 4 per cent in the acid period, would not reduce the effective oral dosage to an appreciable extent. The actual measurements on quinacrine plasma levels in the present experiment showed no cyclic fluctuation in acid and alkaline phases as were observed with santoquine.

One therapeutic implication of the above results is obvious. When toxicity is encountered during the administration of certain anti-malarial drugs, acidification of the urine by ammonium chloride would promote renal excretion of the drug. Effectiveness of such therapy should be good only with drugs of high excretion rate such as chloroquine and santoquine. With a drug like quinacrine which is excreted in the urine to only a slight degree even upon acidification, such therapy should have little value.

SUMMARY

1. The oral administration of ammonium chloride increases the renal excretion of quinacrine, chloroquine and santoquine in individuals on a constant maintenance dose of the drugs. Sodium bicarbonate given in a similar manner causes a retention of these drugs.

2. Changes in renal excretion are reflected in changes in plasma level only when the absolute rate of excretion of the substance is high.

BIBLIOGRAPHY

1. Haag, H. B., and Larson, P. S., Studies on the fate of nicotine in the body; the effect of pH on the urinary excretion of nicotine by tobacco smokers. J. Pharmacol. & Exper. Therap., 1942, 76, 235.
2. Haag, H. B., Larson, P. S., and Schwartz, J. J., The effect of urinary pH on the elimination of quinine in man. J. Pharmacol. and Exper. Therap., 1943, 79, 136.
3. Emerson, K., and Dole, V. P., The renal excretion of atabrine. Malaria Report No. 246, Board for the coordination of malaria studies, 1943.
4. Army Malaria Research Unit. Factors affecting the excretion of mepacrine in the urine. Ann. Trop. Med., 1945, 39, 53.
5. Trager, W., and Hutchinson, M. C., The influence of ammonium ion on the plasma atabrine level and on the urinary excretion of atabrine. J. Clin. Invest., 1946, 25, 694.
6. Brodie, B. B., and Udenfriend, S., The estimation of atabrine in biological fluids and tissues. J. Biol. Chem., 1943, 151, 299.
7. Masen, J. Personal communication.
8. Brodie, B. B., Udenfriend, S., and Dill, W., The estimation of basic organic compounds in biological material; estimation by salt formation with methyl orange. J. Biol. Chem., 1947, 168, 335.
9. Brodie, B. B., Udenfriend, S., Dill, W., and Chenkin, T., The estimation of basic organic compounds in biological material; estimation by conversion to fluorescent compounds. J. Biol. Chem., 1947, 168, 319.
10. Kempton, R. T., Differences in the elimination of neutral red and phenol red by the frog kidney. J. Cell. and Comp. Physiol., 1939, 14, 73.
11. Jailer, J. W., Rosenfeld, M., Zubrod, G., and Shannon, J. A. In press.

PROCEEDINGS OF THE THIRTY-NINTH ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION HELD IN ATLANTIC CITY, N. J., MAY 5, 1947

The Antihemophilic Principle of Normal Plasma. BENJAMIN ALEXANDER, Boston, Mass. (Introduced by S. L. Gargill).

A method devised in our laboratories, for the quantitative measurement of the antihemophilic factor in normal plasma, has been applied to a study of some of its physiological and biochemical properties. The method is based upon the observation, now extended to include a total of 5 hemophiliacs, that the relationship between the logarithm of the amount of plasma added in vitro to hemophilic blood and the logarithm of its clotting time is linear.

The effects of normal plasma on hemophilic blood both in vivo and in vitro are constant and equivalent. The intravenous administration of 150 to 200 cc. of plasma 3 to 4 times weekly to 4 hemophiliacs has proved effective in the prevention of hemorrhage for periods of 6 to 16 months. Intramuscular plasma is much less effective in lowering the clotting time.

The antihemophilic potency of 46 non-hemophilic plasmas was uniform with a standard deviation of 0.3 in terms of an average of 1.0. The antihemophilic factor is largely retained by a Berkefeld filter but passes through a Seitz. The substance is no longer freely present following blood coagulation.

Frozen plasma remains fully potent for over six months but liquid refrigerated plasma (5° C.) loses half of its activity in 5 weeks, 90 per cent in 2½ months. At 37° C. one half is lost in 10 hours, 85 per cent in 24 hours. The rate of deterioration is uninfluenced by platelets or by the admixture of hemophilic plasma, but is much more rapid in whole blood, or normal plasma-whole hemophilic blood mixtures incubated at 37° C. In the hemophiliac 93 per cent of the antihemophilic factor of infused plasma disappears within 13 hours.

Mixtures of normal and hemophilic plasma have a greater antihemophilic potency in vitro than normal plasma alone whereas hemophilic plasma is inert. The enhanced potency is referable to the hemophilic platelets.

These observations are of value both in the clinical management of hemophilia and in further elucidation of the mechanism of blood coagulation.

Mechanisms Underlying Pulmonary and Cardiac Complications of Electrically Induced Convulsions in the Treatment of Psychosis. M. D. ALTSCHULE and (by invitation) K. J. TILLOTSON, Boston, Mass.

Convulsions induced by electroshock are accompanied by maximal forced expiration. During the seizure salivation occurs, and roentgenograms suggest also that bronchial secretion is likewise increased at this time. Maximal inspiration at the end of the seizure causes the aspiration of this material and gives rise to atelectasis more commonly than is suspected. Infection may localize in an atelectatic area and result in abscess formation. Increase in intrathoracic pressure during seizures exerts a force on the outside of the coronary arteries which prevents an increase in difference between the internal and external pressures on the walls of these arteries; brittle coronary arteries are thereby protected. Also, the changes in intrapleural pressure retard passage of blood from the periphery into the heart; this change is reflected in an increase in peripheral venous pressure. As a consequence of diminished venous return the heart becomes smaller than normal and its work is decreased. After the end of the convulsion, the cardiac work is increased, judging by changes in circulation time. The rush of blood from the periphery into the right auricle and great veins after the convulsion gives rise to cardiac arrhythmias which are vagal in type.

The Mechanism of Control of Extravascular Fluid Formation by Normal Human Serum Albumin in Cirrhosis and Idiopathic Hypoproteinemia with Normal Renal Function. S. HOWARD ARMSTRONG, JR., and (by invitation) SAM T. GIBSON, Boston, Mass.

In three patients periods of baseline control have exceeded six months, during which observations on water, salt, and nitrogen balances, plasma volume, protein distribution in serum and extravascular fluids, and differential osmotic pressure measurements yielding the effective osmotic pressure of the serum as against the extravascular fluid have been made.

When the course during control period suggested that the disease process was static for purposes of the experiment, albumin administration was started.

Cessation of formation of extravascular fluid has been observed when the differential osmotic pressure attains the level of normal osmotic pressure for man. Fluid formation recurs when this pressure falls to original levels. The variables unrelated to osmotic pressure do not appear to have first-order significance. In the effect in these patients without significant protein loss in the urine, the amount of albumin required to maintain this differential pressure is far less than the initial amount required to attain it. From the amounts used, together with the nitrogen balances, rough estimates have been made of tissue protein depletion which must be met before blood proteins rise in these patients.

Whereas the albumin has appeared to eliminate the disability of being waterlogged in these selected patients, the studies are not sufficiently wide to permit any conclusions on the effect of therapy on the underlying course of the disease.

*Vascular Spiders and Palmar Erythema in Pregnancy** WILLIAM B. BEAN and (by invitation) MORRIS W. DEXTER and ROBERT C. COGSWELL, Cincinnati, Ohio.

An extension of studies on vascular spiders and palmar erythema which may complicate liver disease has been made to determine the incidence, progress and fate of

* Aided by a grant from the U. S. Public Health Service.

these vascular changes during and after uncomplicated pregnancy. Observations on 274 colored and 178 white women have been made at each routine visit to the Obstetric Prenatal Clinic; 70 colored and 26 white women have been observed shortly after delivery and 6 weeks later. Including the earliest definite lesions, vascular spiders have been found in 65 per cent of white women and 15 per cent of colored women. Palmar erythema was detected in 58 per cent and 32 per cent respectively; and both types of vascular changes existed simultaneously in 43 per cent and 10 per cent. Incomplete data on the time of appearance of lesions indicate that they may begin at any time during pregnancy. Since many patients made their first clinic visit late in pregnancy very few known lesions were seen before the fifth month. In more than two-thirds of the patients the acquired vascular spiders and palmar erythema had disappeared by the 3rd to 5th days following delivery, and a few more had vanished by the time of the 6-week follow-up visit. Studies of liver function and urinary excretion of steroid hormones are in progress.

*Physiological Studies in Congenital Heart Disease** RICHARD J. BING, LEROY D. VANDAM, and FRANK D. GRAY, JR., Baltimore, Md. (Introduced by E. Cowles Andrus).

One hundred eighty patients with congenital heart disease of the cyanotic group were studied. Pulmonary capillary flow was measured using the indirect Fick principle. Formulae for the determination of the volume flow through the pulmonary artery, the systemic capillaries, and through the aorta were devised from results obtained by catheterization of the heart. Similarly, the amount of mixed venous blood perfusing the lung (effective pulmonary blood flow), the size and direction of the intracardiac shunt, and the volume of collateral circulation to the lung were measured.

The effect of a standard exercise test on circulatory and respiratory functions was studied.

In tetralogy of Fallot the intracardiac shunt was directed from right to left. Large collateral circulation to the lung was present in most cases.

The arterial oxygen saturation was found to be related to the percentage of total mixed venous blood perfusing the lung. The systolic pressure in the right ventricle was elevated, and peripheral resistance varied with the logarithm of the systemic flow.

The basal metabolic rate was below normal. During the exercise test the ratio of oxygen consumption per liters of ventilation and the arterial oxygen saturation declined.

The Blalock operation was followed by an increase in the pulmonary capillary flow, the effective pulmonary blood flow, and the arterial oxygen saturation.

In Eisenmenger's complex the pulmonary artery flow was normal. Both systolic and diastolic pressures in the pulmonary artery were elevated. During the exercise test the ratio of oxygen consumed per liters of ventilation increased, and the arterial oxygen saturation declined.

* This work was supported by a grant from the Commonwealth Fund.

The Effect of Ouabain on the Dynamics of the Circulation in Patients with Congestive Heart Failure. RICHARD A. BLOOMFIELD, BERNARD RAPOPORT, J. PERVIS MILNOR, WALTER K. LONG and J. GILMER MEBANE (by invitation) and LAURENCE B. ELLIS, Boston, Mass.

The effect of ouabain upon the circulation in congestive heart failure was studied in a series of patients by utilizing the intracardiac catheterization technique. Cardiac outputs, by the direct Fick method, were determined before and at 1 to 2 hours after administration of ½ to 1 mg. of the drug through the catheter. Simultaneous recordings through Hamilton manometers were made at frequent intervals over the same period of time of the peripheral venous, femoral arterial, and right intraventricular pressures; in addition, where possible, registration of the pulmonary arterial pressure was also done. Changes in heart rate were for the most part of a small order and did not explain observed alterations in other measurements. In those patients with congestive heart failure in whom the administration of ouabain was followed by a significant rise in the cardiac output no consistent or significant pattern of peripheral venous pressure change was observed; in fact, in some of the patients with marked increases in cardiac output the venous pressure was unchanged. In most patients there was an early and at times sustained rise in the systemic blood pressure. The data obtained failed to substantiate the view that the primary effect of the cardiac glucoside was due to a peripheral action on the venous pressure. The significance of the changes observed in the cardiodynamic measurements, including peripheral and right intracardiac pressures, is discussed, and it is concluded that they are consistent with a transfer of blood from the pulmonary and venous pools to the systemic side of the circulation.

High Penicillin Plasma Concentration by the Use of Caronamide, a Compound That Inhibits Penicillin Excretion by the Renal Tubules. WILLIAM P. BOGER (by invitation), CALVIN F. KAY and (by invitation) SYLVAN H. EISMAN and ELMER E. YEOMAN, Philadelphia, Pa.

The reversible and physiologic inhibition of penicillin excretion by the renal tubules following the oral administration of caronamide, 4'-carboxy-phenylmethanesulfonanilide, has recently been described. It has been shown that when penicillin is administered parenterally or orally, the resultant plasma concentrations can be enhanced from two to seven times by reasonable caronamide doses given orally.

This work was stimulated by the necessity of attaining high penicillin plasma concentrations for the treatment of a patient suffering from subacute bacterial endocarditis due to a streptococcus viridans highly resistant to both streptomycin and penicillin. Very high plasma concentrations of penicillin have been achieved (fourteen to forty units of penicillin per cubic centimeter), following the oral administration of caronamide every three hours and the intramuscular injection of 500,000 units of penicillin every three hours. Dose-response curves following the injection of penicillin have been compared in control and caronamide treatment periods in order that the enhancement effect of caronamide could be evaluated. The peni-

cillin plasma concentrations have been correlated with the quantities of penicillin recovered in the urine, the plasma concentration of caronamide, the renal clearance of penicillin, and the glomerular filtration rate as determined by mannitol clearance.

Estimated Hepatic Blood Flow and Bromsulfalein Extraction in Normal Man During the Pyrogenic Reaction. STANLEY E. BRADLEY and (by invitation) NEAL J. CONAN, New York, N. Y.

The present study was undertaken to determine whether hepatic circulatory adjustments and/or hepatic cellular dysfunction develop in man during the pyrogenic reaction. The cardiac output and the renal blood flow increase even when fever is prevented by antipyretic drugs, and, since renal hyperemia is not sufficient to account for the increment in cardiac output, it may be presumed that the hepatic circulation is also increased. Recently it has been found that bromsulfalein (BSP) retention occurs during fever. This phenomenon may be attributed to (1) hepatic ischemia with reduced delivery of BSP to the liver or (2) diminished ability of hepatic cells to remove the dye from blood perfusing the liver.

The hepatic blood flow (EHBF) was estimated as the bromsulfalein clearance and BSP extraction was determined directly using the technique of hepatic venous catheterization. Studies were made upon normal human subjects prior to, and following, intravenous administration of typhoid vaccine.

Within one to two hours after pyrogen was given EHBF increased significantly in nearly every case (300 per cent above the control level on one occasion). Despite hepatic hyperemia and the resulting increase in BSP loading the removal of BSP from the blood decreased by as much as 50 per cent. This effect was apparently attributable to a striking reduction in BSP extraction by the liver. Premedication with aminopyrine prevented fever but did not alter the responses.

On several occasions, the hepatic venous pressure was observed to rise as hepatic hyperemia developed.

Streptomycin and Tuberculous Tracheo-Bronchitis. A Bronchoscopic Study of Healing Lesions. LYMAN A. BREWER III, EMIL BOGEN and ROBERT L. BRERETON, San Fernando, Calif. (Introduced by J. M. Hayman, Jr.).

Visible lesions are especially suitable for the early evaluation of therapeutic effects in tuberculosis. Thirteen tuberculous patients with persistent, severe, tracheal or bronchial granulating or ulcerative lesions were selected from more than a hundred referred for this purpose. They were intensively treated with streptomycin, both intramuscularly and by aerosol, at the San Fernando Veterans Administration Hospital for periods ranging from one week to three months. Biweekly bronchoscopic examinations showed prompt disappearance of the lesions in every case. Only one instance of recurrence of an ulcer in the tracheo-bronchial mucosa has been seen since the treatment has been discontinued. Pulmonary cavities in these patients remained open, and temporary symptomatic improvement in other lesions in the lungs were not sustained. Special clinical and laboratory studies before, during and after the treatment period help clarify the phenomena observed. This study indicates that streptomycin is a valuable therapeutic agent in the treatment of the most severe types of active tuberculous tracheobronchitis.

Streptomycin in the Treatment of Draining Tuberculous Sinuses. BENJAMIN L. BROCK, Oteen, N. C. (Introduced by Arthur M. Walker).

One of the most dramatic results in the treatment of tuberculosis in man has been obtained following the use of streptomycin in draining tuberculous sinuses.

Sixty draining sinuses, proved to be tuberculous either by culture or biopsy, originating for the most part in bone or cartilage in eleven negro patients and one white patient have been treated with streptomycin over a period of from 90 to 150 days.

A daily dose of 1.8 gm. of streptomycin, or 0.3 gm. every four hours was given intramuscularly.

Nine (15 per cent) of the 60 sinuses closed within one week to four weeks and nine (15 per cent) closed within six weeks to eight weeks after streptomycin was begun. It required between ten weeks and twelve weeks for thirty (50 per cent) of the sinuses to close and the remaining eleven (approximately 20 per cent) closed within thirteen to twenty weeks after streptomycin therapy was instituted. Only one sinus remains following surgical drainage but it is almost closed.

The longest time which has elapsed since closure of a sinus is seven months, one week after therapy was begun; the shortest time one month. The average time since closure of all the sinuses is 3.8 months.

Where necrotic bone, or cartilage, as for example in a rib, is removed and where small abscesses under the skin, or large psoas abscesses in the groin are widely drained in conjunction with streptomycin therapy, arrest of the process with closure of the sinus occurs within a relatively short time.

Certain reactions to streptomycin are described and several outstanding clinical signs of improvement are discussed. Photographic illustrations are available.

Analysis of Peritoneal Washings for Protein, Non-Protein Nitrogen and Phosphorus During Studies on Nitrogen and Fluid Balance in Treatment of Acute Uremia by Peritoneal Lavage. HERBERT R. BROWN, JR., JACOB HOLLER and HELEN E. VAN ALSTINE, Rochester, N. Y. (Introduced by Samuel H. Bassett).

A woman of 25 years who developed acute anuria during the course of glomerulonephritis was treated for 21 days by continuous peritoneal irrigation using the method of Fine. The procedure was terminated by death of the patient. During the interval of study it was possible to (a) keep a fairly complete nitrogen balance (b) get rid of severe edema (c) increase the plasma CO_2 from 20 to 60 volumes per cent and (d) reduce the level of NPN in the blood.

A very considerable amount of protein (731 gm.) was recovered from the peritoneal washings. The mechanism of this was thought to be the establishment of partial

equilibrium between the perfusing solution and the patient's interstitial fluid. Increased capillary permeability, believed to occur in acute nephritis, may have been a contributing factor to protein transudation in the early phase of lavage.

The overall picture of the N balance (exclusive of three small stools and losses from the skin) may be tabulated as follows:

NITROGEN BALANCE

Nitrogen Intake		
Whole Blood (Intravenously)	63.3 gm.	
Plasma (Intravenously)	99.6 gm.	
Aminoids (orally)	76.5 gm.	
Special formula (orally)	36.6 gm.	
Total	276.0 gm.	276.0 gm.
Nitrogen Output		
Peritoneal Drainage		
(a) NPN	140.8 gm.	
(b) Protein N	117.0 gm.	
Gastric Suction (Wangensteen)	92.5 gm.	
Urine	4.4 gm.	
Total	354.7 gm.	354.7 gm.
Difference (Nitrogen loss)		78.7 gm.

Rates of Turnover of Radiosodium in the Blood and Urine of Normal Subjects and Patients with Congestive Heart Failure. GEORGE BURCH and PAUL REASER, New Orleans, La.

The rates of turnover of sodium in the blood and urine of 10 normal subjects, 12 patients with various degrees and stages of congestive heart failure and 6 patients with miscellaneous clinical states were determined for a period up to 120 minutes with Na^{24} ($t_{\frac{1}{2}} = 14.8h$). The subjects were on a regular hospital or a low salt diet (1.7 gm. NaCl). Most of the patients with congestive heart failure were receiving the usual therapy for congestive heart failure without the use of diuretics.

The Na^{24} was injected in one antecubital vein and blood was collected from the other at 5–10 second intervals for 3 minutes. The intervals between collections were increased as the study progressed. Urine was collected simultaneously and separately from the pelvis of each kidney by means of catheters. The urine was collected at intervals varying from a few seconds to a few minutes, depending upon the rate of urine flow.

The results showed a very rapid rate of Na exchange between the blood vessels and tissue spaces, being several times 7,000 grams Na per day. The rate of diffusion was so rapid that the rate of mixing and circulation time were too slow to permit accurate measurements of the Na exchange, much of the Na having already escaped from the blood before a complete recirculation. The nature of the curves of Na^{24} level in the blood were quite different from those reported for dogs in which samples were collected from arteries. The differences in blood Na levels between the normal and congestive heart failure subjects could be accounted for by the volume of the water in the Na compartments.

The urine concentrations of Na of the normal subjects were essentially the same as for the blood. That of the patient with congestive heart failure was uniformly lower but varied with the phase of the congestive heart failure and the Na in the diet. The "Na clearances" in the patients with congestive heart failure were lower than those in the normal subjects. The Na^{24} appeared in the urine of all subjects within 1.5 to 3.5 minutes after injection.

The data show an impairment of sodium excretion in congestive heart failure due largely to renal dysfunction.

Miliary and Meningeal Tuberculosis Treated with Streptomycin. PAUL A. BUNN, Washington, D. C. (Introduced by Thomas McPherson Brown).

From May 1946 until February 20, 1947, 60 cases of proved meningeal and miliary tuberculosis, or a combination of both, have been treated in 38 Veterans Administration hospitals. The number of cases in each group, the death and survival rates according to the time treated, and the number who are living after completion of an assigned course of streptomycin are shown in the figure.

Disease	Number of cases	Dead, 27, following treatment days		Living, 33, following treatment days		Treatment "completed"
		0–7	15–120	10–30	30–125	
Meningitis	24	8	5	8	3	0
Miliary	17	0	2	2	10	3
Meningitis and miliary	19	5	7	0	4	3
Totals	60	13 (22%)	14 (23%)	10 (17%)	17 (28%)	6 (10%)

Only those cases falling into the classification of acute disseminated miliary tuberculosis and those with classical signs of meningitis are included. Without specific antibacterial therapy, the expected mortality of each group would approximate 100 per cent.

Of the 6 cases who have completed treatment to date, 4 are to all intents and purposes normal—both clinically and psychologically. The fifth continues to have active pulmonary tuberculosis and the sixth has developed irreparable brain damage.

The clinical improvement, the laboratory findings and the roentgenographic changes in all cases during the period of therapy have been recorded and are extremely impressive in most instances. Complete x-ray clearing of all miliary lesions in the lungs has been demonstrated on occasions.

Many evidences of toxicity to streptomycin have been recorded. Among these are three cases who developed complete deafness and one in whom there was bone marrow depression.

The dosage of streptomycin has varied from 1.2 gm. to 4 gm. daily given in divided doses. The duration of treatment has been variable. The material has been continuously administered for as long as 160 days, but in most,

if the patient survives, therapy was stopped at the end of 120.

The Effect of Age on Phosphate Fractions in the Heart of the Rat. GUS G. CASTEN,* and M. F. MASON, Dallas, Texas. (Introduced by Tinsley R. Harrison).

A comparison has been made between young adult male rats (about 3 months old) and elderly male rats (about two years old) in regard to the content of the heart in various phosphate fractions. No significant difference was found in regard to the inorganic phosphate or the adenosine triphosphate. Striking differences were observed in the creatin phosphate fraction which was about twice as great in the young as in the older rats. Since the creatin phosphate constitutes the reserve from which adenosine triphosphate, the direct source of energy, may be derived under conditions of stress, the findings are in general agreement with the conception that aging is associated with a decline in the reserve power of the heart. The reasons why heart and skeletal muscle (as judged from the work of others) differ in respect to the effect of age on creatin phosphate content are not clear.

Further studies dealing with the effect of experimental heart failure on the various phosphate fractions are in progress.

Sulfathiazole as a Substitute for Inulin in Determining the Glomerular Filtration Rate. DON W. CHAPMAN, and S. A. PEOPLES, Houston, Texas, (Introduced by James A. Greene).

An accurate method for measurement of glomerular filtration has been developed, by which a larger number of patients can be studied more quickly, and is simple enough for routine clinical studies.

Sulfathiazole was given orally and its clearance compared with that of inulin. Urine specimens were obtained by catheterization at twenty or thirty minute intervals, and blood samples were drawn at the mid-points of each interval. The urine and plasma were analyzed for sulfathiazole by the method of Bratton and Marshall and the plasma albumin by the method of Greenberg. The free plasma sulfathiazole was calculated from the total plasma sulfathiazole, from the concentration of plasma albumin by means of the adsorption formula

$$\frac{\text{Free Sulfa}}{\text{Total Sulfa}}=\frac{1}{K\times \text{Albumin Conc.}+1}, \quad \text{where} \quad K=0.45.$$

The K value was obtained from simultaneous clearance studies of inulin and sulfathiazole in humans.

Maximum deviation of simultaneous clearance of inulin and sulfathiazole in 25 patients was 11 per cent, and an average deviation of 6 per cent. The data show that sulfathiazole clearance is as reliable as inulin clearance in patients with normal and with reduced renal function.

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* Done during the tenure of a Life Insurance Medical Research Student Fellowship.

Study of Standard Metabolism in Relation to Degenerative Processes in the Aged. MARGARET CHIEFFI, St. Louis, Mo. (Introduced by William B. Kountz).

A detailed study was made on about 500 patients over 40 years of age. Some of these were followed over an extensive period, up to eight years, and were studied repeatedly.

The individuals were either residents in a chronic disease hospital or were visitors to a private clinic. The result of the studies showed the individuals to form a series varying from those with earliest signs of degeneration (which on repeated study were found to be progressive) to those with advanced degenerative changes.

Many interesting variations were noted in a study of the different tests. Changes in the basal metabolism seemed to be the most significant and the results will be considered.

In those individuals with early degenerative changes the metabolism was usually low, varying from -10 to -28 and averaging about -15.

The individuals with advanced degenerative changes and with residuals of diseases such as coronary artery disease, intracranial lesions etc. frequently show an elevated basal metabolic rate not only as compared with the first group but even compared with the normal standard. Repeated studies in these individuals showed a rising metabolic rate.

Results of attempts to modify the metabolism by feeding experiments and administration of hormones will also be discussed.

Rapid Quantitative Assay of Proteolytic Enzyme Inhibitors in a Fibrinogenolytic System and the Significance of the Plasma Inhibitor in Experimental Peritonitis in the Dog. DONALD G. C. CLARK, New Haven, Conn. (Introduced by John S. Lockwood).

Preliminary investigation of experimental peritonitis in the dog has suggested that the level of proteolysin inhibitor in the plasma is a factor in the ability of the animals to survive the disease. In this work 29 pairs of dogs were subjected to peritonitis; one of each pair was prepared by the daily injection of protease for 5 days prior to operation in an attempt to raise the level of proteolysin inhibitor, as suggested by the work of Grob, while the other of each pair was used as a control. The difference in mortality was significant, 5 dogs dying from the prepared group (17.2 per cent) and 20 from the control series (68.9 per cent).

Since the difference in mortality appeared to be a result, at least in part, of the much more marked production of fibrin around the abscess in the treated animals, as shown at autopsy, it was felt that the effect of the proteolysin-inhibitor system on the stability of fibrinogen might be the significant factor. This, together with the fact that fibrinogen is highly sensitive to the action of proteolysins such as trypsin, led to the development of an assay method employing fibrinogen as the substrate.

In the method serial dilutions of serum from the animal are allowed to react with a known amount of trypsin for a fixed period to allow the proteolysin and proteolysin inhibitor to react together. Then a standard quantity of

lysin-free fibrinogen is added. The formation of a clot upon addition of thrombin, after a standard period of incubation at 37 C., indicates that the trypsin has been inhibited, and affords a clear-cut end-point; no clot forms if the quantity of inhibitor has been insufficient to block the added trypsin. Temperature, pH, and timing of the various phases of the reaction must be carefully controlled.

The normal dog shows a level of inhibitor equivalent to from 300 gamma to 450 gamma crystalline soy bean trypsin inhibitor (Kunitz) per ml. serum. Following injections of protease, however, the inhibitor level at first falls and then returns to the initial level with increasing speed on each subsequent injection. The length of the recovery period decreases from about 9 hours after the initial injection to about 1 hour after the fifth injection. Also, in most animals the titre increases gradually with each successive injection to about double the original value. In about one-quarter of the animals studied this absolute increase did not develop.

It seems probable that a stable equilibrium between plasma protease and its inhibitor is a factor in the effective deposition of fibrin within the peritoneal cavity, and in recovery from peritonitis.

The Effect of Castration and Di-ethyl Stilboestrol in a Patient with Myelophthisic Anemia Secondary to Carcinoma of the Prostate. Robert R. Commons (by invitation) and Maurice B. Strauss, Framingham, Mass.

Severe myelophthisic anemia may be the first and outstanding manifestation of carcinoma of the prostate with widespread skeletal metastases. Three such cases have been observed, in none of which were prostatic symptoms presenting.

The first two of these patients, observed prior to the introduction of castration and female sex hormone administration in the treatment of prostatic cancer, died within a few months of diagnosis, with their anemia controlled only by blood transfusion. The third patient was castrated after a three weeks intensive course of liver and iron therapy had been accompanied by a further decline in blood values. Five mg. of di-ethyl stilboestrol was then administered by mouth daily. No other treatment was employed. Bone pain, ascribable to metastatic skeletal involvement, disappeared within four weeks. No reticulocyte increase occurred but a definite slow rise in red blood cells and hemoglobin occurred from an original level of 1,990,000 erythrocytes per cu. mm. and 5.1 gm. of hemoglobin per 100 cc. to values of 3,530,000 and 11.0 gm. two months after the institution of treatment. Nucleated red blood cells, which had been present in the proportion of 1 to 100 leukocytes, disappeared from the peripheral blood.

Since neither castration nor the administration of diethyl stilboestrol has been reported to produce increases in red blood cells and/or hemoglobin in man or animals, it appears probable that one or both of these procedures so affected the cancerous metastases in the bone marrow of this patient that more normal hematopoiesis took place. It is of interest that in spite of the striking change in the blood values, x-ray examination failed to show any change in the extent or severity of the bone changes.

The Effect of the Upright Posture upon Hepatic Blood Flow in Normal and Hypertensive Human Subjects. James W. Culbertson (by invitation), Robert W. Wilkins, Franz J. Ingelfinger and Stanley E. Bradley, Boston, Mass.

Hepatic blood flow was estimated in seven normal and twelve hypertensive subjects by means of the bromsulphalein clearance technique with hepatic venous catheterization. Arterial pressure and pulse rate were recorded at two-to five-minute intervals throughout the two-hour test.

Each subject was placed supine on a tilt-table and studied first in a horizontal position, then after being tilted upright (75°), and then in the horizontal position again. Serial pairs of peripheral and hepatic venous blood samples were drawn at five- to ten-minute intervals, three or more for each tilt-table position unless syncope intervened during the upright study.

Values for estimated hepatic blood flow in both groups of subjects before tilting fell within normal range and averaged 1400 ml. per minute per 1.73 sq. m. of body surface. After the tilt to upright the values decreased sharply by 15 to 60 per cent, with an average approaching 40 per cent and no significant difference observed between normal and hypertensive individuals. Following return of the table to the horizontal position hepatic blood flow increased, rising toward or above the pre-tilt control rate.

These results are interpreted as indicating that splanchnic vasoconstriction occurs in the upright position, since the decreases in blood flow could not be accounted for by significant changes in blood pressure.

An Objective Method for Evaluating the Anti-Asthmatic Effect of Several Sympathomimetic Amines in Man. John J. Curry, Boston, Mass. (Introduced by Francis C. Lowell).

The effectiveness of various anti-asthmatic compounds usually has been determined by analyzing the subjective response of large groups of patients. Measurements of increase in vital capacity following the administration of various drugs during the acute asthmatic attack have also been made. Both types of study, however, are subject to unaccountable variations and are difficult to evaluate.

It has been shown that a quantitative decrease in the vital capacity may be produced in certain asthmatic subjects, during periods when they are relatively asymptomatic, by the parenteral administration of histamine and acetyl-beta-methyl choline. Various sympathomimetic amines, anti-histamine compounds and anti-cholinergic agents protect these subjects against the reduction in vital capacity in a characteristic fashion. These findings suggested that the degree of such protection, afforded by various anti-asthmatic drugs, might furnish an objective method for assaying their effectiveness in the treatment of asthma.

In the present study, using this method, several sympathomimetic amines were compared with ephedrine sulfate. Only one compound, beta-o-methoxyphenyl isopropyl methylamine, compared favorably with ephedrine. This was of interest since the compound has little pressor

or C.N.S. stimulating effect. Clinical studies in a group of asthmatic patients supported the results of the assay and the validity of the method.

Relative Importance of Tonicity and of Extracellular Volume in Salt Depletion Shock. T. S. DANOWSKI, A. W. WINKLER and J. R. ELKINTON, New Haven, Conn.

Studies have been conducted to determine whether salt depletion shock is mediated primarily through hypotonicity or through a decrease in the volume of the extracellular fluid. Previously reported experiments indicated that in the salt depleted animal restoration of extracellular and plasma volume without replacement of salt failed to improve the circulation. The results suggested that the hypotonicity and associated cellular overhydration had a deleterious effect *per se*.

In the present experiments hypotonicity of body fluids in the presence of a normal salt content was produced in 3 nephrectomized dogs by the infusion of glucose solution. Changes in the electrolyte and water content of plasma, extracellular fluid, and of cells were calculated and alterations in the hemodynamics (cardiac output, circulation velocity, and mean arterial pressure) were measured.

Those animals in whom body fluids had been diluted resembled those depleted of salt in that hypotonicity was present. They differed, however, in that the extracellular volume was expanded rather than contracted, and despite the hypotonicity, the circulatory efficiency was unimpaired or improved.

In 2 other animals salt depletion shock was produced by the Darrow-Yannett technique. Subsequently a diuresis was induced by intravenous urea solution and the hypotonicity which resulted from the salt depletion was corrected. The extracellular fluid volume declined further, however, and despite the restoration of isotonicity the cardiovascular function did not improve.

It is concluded that within certain limits both tonicity and extracellular volume, *i.e.*, the amount of salt present, must condition the development of salt depletion shock.

Streptomycin in the Treatment of Pulmonary Tuberculosis. NICHOLAS D. D'ESOPO, STANTON T. ALLISON, BENJAMIN L. BROCK, W. A. CASSIDY, and ARNOLD SHAMASKIN, Baltimore, Md. (Introduced by James Bordley III).

120 cases of pulmonary tuberculosis were treated for 4 months with 1.8 grams of streptomycin intramuscularly daily. Patients were selected on the basis of stationary or progressive lesions with an important exudative component during a pre-treatment observation period of at least 3 months. The majority were far-advanced.

Mean blood levels of 18 micrograms and 10 micrograms of streptomycin per cc. after 1 and 4 hours respectively were obtained.

Decline in fever, increased appetite, gain in weight, and a marked decrease in sputum amount were consistently observed.

Resolution of lesions, to a greater or lesser extent, was noted in 77 per cent. In 18 per cent the degree of resolution was striking. No new lesions developed under treatment, nor did established lesions progress. The most favorable effect was seen in cases of predominantly exudative disease. Cavities tended to become smaller, but unequivocal closure was infrequently observed.

Sputum or gastric cultures became negative in 30 per cent of patients.

Toxicity was manifested by subjective vertigo in 90 per cent, an abnormal urinary sediment in 50 per cent, and a transient rash in 10 per cent. Treatment was discontinued in 3 cases.

After 6 weeks the tubercle bacilli of 27 per cent showed in vitro resistance to concentrations of streptomycin obtained at this dosage; and at 4 months 53 per cent developed resistance. In the great majority resistance developed abruptly.

Streptomycin is most useful in controlling symptoms and the recent exudative lesion. It is suppressive rather than definitive therapy. The development of resistant organisms is a disadvantage since there is an inverse correlation between resistance and therapeutic response.

Cardiac Oxygen Metabolism. J. E. ECKENHOFF, J. H. HAFKENSCHIEL and M. HARMEL, Philadelphia, Pa. (Introduced by Francis C. Wood).

In spontaneously breathing dogs lightly anesthetized with nembutal, coronary blood flow was measured by the bubble flowmeter technic and cardiac metabolism determined by a method previously described. In an attempt to elucidate the inter-relationships of coronary flow, arterial pressure, cardiac output, cardiac efficiency and cardiac oxygen metabolism, three groups of experiments were performed in which (1) arterial pressure, (2) cardiac output or (3) arterial oxygen content was primarily altered. In (1) and (2) coronary flow consistently followed cardiac oxygen consumption and in (3) coronary flow was found to increase as needed to meet cardiac oxygen requirements. The data indicate that the coronary vessels, like the cerebrals, possess an effective intrinsic control in relation to the metabolic requirements of the tissue supplied. Cardiac efficiency tended to vary directly with cardiac output and inversely with arterial blood pressure. Since coronary venous blood under the conditions of these experiments normally contained only 4 to 6 vol. per cent oxygen, there is obviously little room for removing appreciably larger amounts of oxygen. Agents intended to accomplish this are therefore open to question. The heart was found to be safeguarded from dangerous anoxia by three mechanisms: (1) decreased tonus in the coronary vessels, (2) diversion of a relatively larger fraction of the cardiac output to the coronary circulation, (3) decreased cardiac work with a resultant decrease in oxygen demand.

The Administration, Utilization and Excretion of a Mixture of Amino Acids in Man. R. D. ECKHARDT and T. L. MURPHY (by invitation), and C. S. DAVIDSON, Boston, Mass.

The 10 per cent solution of the ten "essential" amino acids used in these studies was prepared by complete acid hydrolysis of casein, was rendered free of aspartic and glutamic acids, and was fortified with dl-tryptophane, dl-

methionine and glycine. In over 150 injections reactions were rare, and none of a pyrogenic nature was observed. Injections of 500 cc. (50 gm. amino acids) in 10–15 minutes rarely induced vomiting, although transient nausea occurred in approximately one-fourth of these cases. At a slower rate (one hour), even nausea was rare. Thrombosis of the infusion vein did not occur.

The blood amino acid level returned to within normal limits by four hours after the infusion, although it was often slightly above the pre-injection level. During this time, the maximum loss of the injected amino acids occurred (average 8 per cent). Urinary amino nitrogen excretion did not correlate with the rate of infusion, but was roughly proportional to the amount given and to the total 24-hour urine nitrogen.

Comparison was made between the percentage composition of the essential amino acids administered and excreted (microbiological assay). Lysine, methionine, phenylalanine and valine were excreted in approximately the same percentages as administered; histidine, threonine and tryptophane, in two to three times greater percentages; and arginine, leucine and isoleucine proportionately less.

The Restoration of Thermal Balance During Acclimatization to Work in a Hot Dry Environment. LUDWIG W. EICHNA, (by invitation) CHARLES R. PARK, NORTON A. NELSON, EDWARD D. PALMES, STEVEN M. HORVATH and WALTER B. SHELLEY and WILLIAM B. BEAN, Fort Knox, Ky.

When man first works in a hot environment his body temperature rises to abnormally high levels and disability often results. When acclimatized to the heat he performs the same work easily and with a lower, or normal, temperature. The methods of partitional calorimetry were used to determine the mechanisms responsible for this reduced heat retention.

Thermal balances were determined in 3 nude men as they acclimatized to work in a hot, dry (desert type) environment.* With acclimatization the bodily heat content fell; with deep tissues heat content reaching normal values but that in the peripheral tissues (skin) remaining above normal. Approximately 90 per cent of the total decrease in heat retention with acclimatization was due to increased evaporative cooling (increased secretion and evaporation of sweat); the remaining small decrease (10 per cent) resulted from a slight fall in metabolic heat production. Because of the increased evaporative cooling the skin temperature fell with acclimatization. This led to increased heat gains from the environment by convection and radiation as acclimatization developed. These increased external heat gains amounted to approximately 50 per cent of the increase in evaporative cooling and to that extent decreased the efficiency of the increased evaporative cooling in lowering the body temperature. However, the lower skin temperature promoted heat loss from deep to peripheral tissue by conduction and increased the cooling gradient for the blood brought to the skin. This permitted a reduced peripheral blood flow to the skin and thereby a more stable general circulation.

* Dry bulb temperature 102° F., wet bulb temperature 80° F.

The Mechanism of Blood Destruction in Congenital Hemolytic Jaundice. CHARLES P. EMERSON, JR., and SHU CHU SHEN (by invitation), THOMAS HALE HAM and WILLIAM B. CASTLE, Boston, Mass.

Twenty-nine patients with congenital hemolytic jaundice were studied with particular reference to the nature of the red cell abnormality and the role of the spleen in this hemolytic syndrome. When subjected to sterile incubation *in vitro,* their red cells exhibited abnormally prompt increase in osmotic and mechanical fragilities. In certain individuals with minimal signs of hemolytic disease, who were members of the affected families, this increased mechanical fragility and susceptibility to incubation were the only evidences of red cell abnormality.

Eleven patients with congenital hemolytic jaundice were subjected to splenectomy. Red cells derived from the peripheral blood and from the splenic pulp were compared with respect to their osmotic and mechanical fragilities, and response to incubation. Similar studies were carried out on five patients subjected to splenectomy for conditions other than congenital hemolytic jaundice. Their red cells behaved like normal red cells. However, in all the cases of congenital hemolytic jaundice, the splenic pulp contained a high proportion of red cells that were markedly susceptible to hemolysis in hypotonic solutions and to mechanical trauma when compared to those in the peripheral blood. Post-splenectomy studies of the peripheral blood indicated a rapid disappearance of that portion of the heterogenous red cell population previously exhibiting the most marked increase in osmotic fragility and a return of the mechanical fragility of the blood nearly to normal.

Five patients were given several days before splenectomy massive transfusions of compatible blood possessing immunologic properties which distinguished it from the blood of the recipient. Employing selective agglutination technics it was ascertained that, in contrast to the patient's red cells, the osmotic fragility of these donor cells, including those separated from the splenic pulp, had remained unaltered. Finally, the ratio of patient's cells to donor's cells was found to be distinctly greater in the splenic pulp than in the peripheral blood, suggesting that selective retention of the patient's red cells had occurred. These data indicate that the hemolytic activity of the spleen, in congenital hemolytic jaundice, may derive solely from the fact that this organ serves as a locus of "erythrostasis," a process which *in vitro* demonstrably results in the rapid deterioration of the inherently defective red cells characteristic of the disorder.

The Effect of Diabetes and Insulin on Glucose Tm. SAUL J. FARBER and EUGENE Y. BERGER (by invitation), and DAVID P. EARLE, JR., New York, N. Y.

A high renal threshold for glucose in diabetics has generally been attributed to a low glomerular filtration rate in the presence of a normal glucose reabsorptive capacity. Glucose Tm, or maximum amount of glucose that can be reabsorbed per unit of time, is quite constant

for an individual under standard conditions. Smith *et al.* found the average glucose Tm of normal women to be 303 mgm. per minute ($\sigma = 55.3$). Glucose Tm's determined in normal subjects in this laboratory have fallen within this range. Observations on a limited number of middle aged female diabetic patients have shown an average glucose Tm of 433 mgm. per minute (Range = 401 to 460). The glomerular filtration rates of these patients were normal. The average ratio of filtration rate to glucose Tm was 0.270 as compared to the normal value of 0.395 ($\sigma = 0.0617$).

The continuous intravenous administration of small amounts of insulin to both normal and diabetic subjects resulted in a decrease in glucose Tm in all but one experiment. The average glucose Tm during insulin administration was 10 per cent under control values. Single injections of insulin resulted in transient decreases in glucose Tm of similar magnitude.

Studies on the Rate of Excretion of Water and Electrolyte by Young Infants. Gladys J. Fashena, Dallas, Texas.

Normal infants in the first six months of life, when given isotonic sodium chloride solution parenterally at the rate of 1 per cent of body weight per hour for five hours, excrete the excess loads of water, sodium, and chloride more slowly than adults. In twenty-four hours the average sodium excretion is 75 per cent of the load and the average chloride excretion 91 per cent of the load. The velocity quotients for sodium range between 0.03 and 0.06, for chloride between 0.04 and 0.07 and for water between 0.02 and 0.1. Two infants, recently recovered from dehydration and acidosis, show further impairment of their ability to excrete electrolyte. In these the velocity quotients of sodium and chloride were in the neighborhood of 0.01 and 0.02 respectively and both subjects became slightly edematous during the experiment. It is not known how frequently this type of response follows an episode of dehydration and acidosis and further studies on this point are in progress.

Methemoglobin Reconversion in the Erythrocyte. Clement A. Finch, Howard A. Eder, and Ralph W. McKee, Boston, Mass. (Introduced by George W. Thorn).

Hemoglobin in solution is spontaneously converted to methemoglobin. In the normal erythrocyte this is prevented by a system deriving its energy from carbohydrate metabolism. Studies of a patient with congenital methemoglobinemia have shown an intrinsic defect of the erythrocyte with complete failure of the normal reconversion mechanism. In the absence of cell reconversion, reducing substances appear to regulate the hemoglobin-methemoglobin equilibrium. The role of reducing substances was studied in this patient. It was also observed that methylene blue was able to activate in this patient the cell reconversion mechanism and the immediate and long term effects of methylene blue were studied. The effectiveness of ascorbic acid and methylene blue therapy has been evaluated in different etiological types of methemoglobinemia.

*The Effectiveness in Experimental Syphilis of Penicillin in Peanut Oil-Beeswax Given in Sixteen Daily Injections.** William L. Fleming, Boston, Mass.

Seventy male rabbits inoculated intratesticularly with the Nichols strain of *T. pallidum* were proven to be syphilitic by demonstration of typical spirochaetes from testicular chancres. Eight weeks after inoculation they were treated with sixteen daily intramuscular injections of calcium penicillin suspended in peanut oil containing 4.8 per cent of beeswax by weight volume. Ten rabbits were treated at each of seven total dosage levels: 250, 500, 1,000, 2,000, 4,000, 8,000, and 16,000 units per kilogram. The concentration of penicillin had to be varied in order to administer the different dosages and was of necessity lower than that of the Romansky Formula. Proof of cure or treatment failure was determined by the dark-field examination of chancres or lesions suggestive of infectious relapses, and by lymph node transfers six months after treatment.

Results indicate that the C.D./50 (minimal dose necessary to cure 50 per cent of rabbits) was between 1,000 and 2,000 units per kilogram. This indicates effectiveness in the same order of magnitude as samples of a crude commercial penicillin and of crystalline penicillin G previously assayed after administration in intramuscular injections of aqueous solution every three to four hours for ninety-six hours.

Studies on the Relation of the Serum Albumin to the Formation of Bone Matrix. Anne P. Forbes, Boston, Mass. (Introduced by Fuller Albright).

Osteoporosis is defined as that form of demineralized bone where the defect is in laying down the bone matrix. It has certain clinical characteristics which make its differentiation from other forms of demineralized bone certain. A case is presented with all the characteristic criteria of this disease in which the usual causes (post-menopausal state, disuse, Cushing's Syndrome, malnutrition, et cetera) were not present. The studies include metabolic balances of calcium, phosphorus and nitrogen in relation to the levels of the serum proteins. Various therapeutic regimens were tried. These included therapeutic abortion, plasma by vein, serum albumin by mouth and by vein, high and low protein diets, large doses of Stilbestrol, and Vitamin A. The evidence suggests that bone matrix formation is influenced by the serum albumin level.

Chemical and Electrophoretic Analyses of the Tissue Proteins in Shock from Tourniquet Injury or Burns. Charles L. Fox, Jr., and Dan H. Moore, New York, N. Y. (Introduced by A. R. Dochez).

Experiments were performed to test whether plasma proteins accumulate in tissues traumatized by tourniquetting or burning. Single hind legs of mice were injured by the application and release of tight tourniquets or scalding. When swelling was greatest and shock profound, both hind legs were amputated at the groin and the entire leg analyzed for water and total nitrogen.

* Done under an U.S.P.H.S. Research Grant.

For electrophoretic analysis, similar legs were extracted in chilled buffer.

Injured legs did not contain more protein than contralateral, uninjured legs. If the fluid gained by the injured legs were chiefly plasma, approximately 10 to 20 per cent (20 to 50 mg.) more protein might be expected.

Electrophoretic analyses of the injured leg fluid disclosed no component with the mobility of albumin. Instead a faster, new component appeared; this was resolved from plasma albumin in artificial mixtures. In the ultracentrifuge, its sedimentation constant was greater than albumin.

These chemical, electrophoretic, and ultracentrifugal analyses dispute the view that plasma proteins accumulate in tissues injured by tourniquetting or scalding.

The Ventricular Gradient in Fever. A. S. FREEDBERG and M. J. MCMANUS, Boston, Mass. (Introduced by H. L. Blumgart).

Published studies of the electroardiographic changes during infections stress the occurrence of ST and T wave abnormalities. In these reports, however, it is often impossible to distinguish the effects of fever per se from those of myocardial changes due to many other factors commonly present in infectious disease. The method of Wilson *et al.* for determining ventricular gradients was therefore applied to 3 standard limb lead electrocardiograms obtained during fever induced by intravenous injection of typhoid vaccine in 9 human subjects.

The ST segment depression and T wave inversion occurring in typhoid vaccine fever are (a) primary and (b) secondary. The latter may be related to increased rate and/or a change in heart position. The former resemble the changes observed after digitalis administration and indicate a physiologic alteration in the myocardium related to the induced fever. In two patients pronounced shift in ventricular gradient direction occurred in the chill and flush phase. This indicates a local alteration in duration of the excited state, similar to that observed in local myocardial ischemia. These alterations in ventricular gradient consequent to fever must be considered in the interpretation of T wave and ST segment changes in febrile states, both endogenous and physically induced.

Assay of Urinary Estrogens by Ultraviolet Absorption Spectrophotometry. HARRY B. FRIEDGOOD and (by invitation), JOSEPHINE B. GARST, Pasadena, Calif.

Previous studies have demonstrated that ultraviolet absorption spectrophotometry can be applied to the quantitative determination of pooled or single samples of crystalline estrone, estradiol and estriol by virtue of the type of absorption curves and concentration-extinction relations which these hormones exhibit under controlled experimental conditions. Further investigations with this quantitative technic have disclosed that current chemical methods do not effect a clean-cut separation of the androgens from the estrogens or of estriol from the estrone-estradiol fraction. The significant losses of estrogens involved in these manipulations have been difficult, if not impossible, to estimate accurately because of the relative lack of sensitivity of the colorimetric and bioassay methods of quantitation as compared with that of ultraviolet absorption spectrophotometry. On the basis of these findings, a new method has been developed for the accurate separation and assay of the three estrogens in aqueous-alcoholic solution. A study has been made of the factors involved in the application of ultraviolet absorption spectrophotometry to the extraction and separation of the estrogens from urine—with particular reference to the removal of the background material which interferes with the quantitative reading of the estrogen curves.

The Relationship between Calcium and Digitalis Glycoside in Respect to Their Action upon the Embryonic Duck Heart. MEYER FRIEDMAN and (by invitation) RENÉ BINE, JR., San Francisco, Calif.

The embryonic duck heart was found to be similar to the mammalian heart in its response to excess digitalis glycoside (Lanatoside C.), in that it reacted to the presence of the latter drug by (1) acceleration of rate, (2) increased force of contraction and (3) appearance of arrhythmia. This particular property of the embryonic heart, together with its ability to beat weakly but persistently in the complete absence of calcium or in an excess of the latter, allowed an unique opportunity for the study of the relationship between calcium and digitalis glycoside in their effects upon the heart. The absence of an extrinsic nerve supply to the embryonic heart (at stage of development, used) allowed such a study to be strictly of the cardiac musculature, alone.

The completed studies indicated that the action of calcium alone was completely dissimilar to that of digitalis, irrespective of the concentration of the former. Furthermore, it was found that the calcium ion and the digitalis glycoside employed, bore no synergic relationship to each other in respect to their individual or combined effects upon the heart. Finally, it was discovered that the characteristic action of digitalis was not inhibited by the complete absence of the calcium ion.

Arterial Pressure Pulse Waves in a Patient with Coarctation of the Aorta. MORTON GALDSTON (by invitation) and J. MURRAY STEELE, New York, N. Y.

Arterial pressure was recorded with Hamilton manometers simultaneously in radial and femoral arteries of a middle aged patient suffering from coarctation of the aorta. The usual pressure differences were observed (radial 215–225/100 mm. Hg; femoral 150/100 mm. Hg).

Of greater interest than these differences was the remarkable similarity between pulse waves from the left radial and left subscapular collateral artery, in spite of entirely different physical arrangements of the arterial tree distal to the points from which these pressure waves were recorded, as observed at autopsy. The radial arteries were of normal origin and distribution. The enlarged, tortuous subscapular arteries joined enlarged 5th and 6th intercostal arteries which entered the aorta below the coarctation. The main collateral vessels emptied into enlarged 3rd and 4th intercostal arteries just

below the coarctation, the lumen of which measured 0.5 cm.

Since variations in form of pressure curves in the arterial tree are considered to be influenced partly by waves reflected from distal arterioles and to a lesser degree by standing arterial waves, the similarity of the radial and collateral artery pulse waves is difficult to explain. A similar form of pulse wave has been demonstrated in another patient with coarctation of the aorta and in patients with hypertension when the artery was partially constricted above the point of recording.

Studies of the Relationship of Niacin and Tryptophane in Human Metabolism. GRACE A. GOLDSMITH and (by invitation) HERBERT P. SARETT, New Orleans, La.

Metabolic balance studies were conducted for six weeks in three persons on corn or wheat diets, which provided 38 or 43 gm. of protein, 5.5 or 6.0 mg. of niacin, 0.3 or 0.5 gm. of tryptophane, and about 2500 calories, 0.7 mg. of thiamine and 0.5 mg. of riboflavin daily (calculated). The corn diet included 190 gm. of degerminated corn meal and grits and 60 gm. of white unenriched flour; the wheat diet, 250 gm. of the white flour. The urinary excretion of riboflavin and thiamine decreased rapidly on the basal diets and remained low throughout the experiment, while that of niacin was unchanged. The daily output of N^1 methylnicotinamide was 2 mg. or less on the wheat diet and decreased further on the corn regimen. After ten days of the basal corn or wheat diet, 5 gm. of dl-tryptophane was administered daily for 8 days. This resulted in an increased daily excretion of 6 to 12 mg. of N^1 methylnicotinamide.

A test dose of niacin, thiamine, and riboflavin was given at the end of the tryptophane experiment. The urinary excretion of N^1 methylnicotinamide was normal, of riboflavin, low normal and of thiamine, very low in two and normal in one patient. No signs of vitamin B complex deficiency developed and no significant change in serum proteins or in the blood picture occurred during this study.

Findings suggest that tryptophane is an important precursor of niacin in humans and may contribute to the body stores of this vitamin.

Studies of Immunity in Brucellosis: The Bactericidal Action of Human Blood Against Brucella. WENDELL H. HALL (by invitation) and WESLEY W. SPINK, Minneapolis, Minn.

The information available concerning the role of humoral antibodies in brucellosis is confusing. The present study has revealed that the killing action of normal human blood for Brucella does not require the presence of leukocytes. The effective antibody in serum is heat-stable, and complement is necessary for its optimum bactericidal action on Brucella. *Br. abortus* (16 strains) was more readily killed than *Br. suis* (3 strains) or *Br. melitensis* (1 strain).

Bactericidal tests were performed by adding serial dilutions of Brucella to standardized amounts of fresh serum and incubating the mixtures for 24 hours. No killing of *Br. abortus* was obtained with sera from 10 infants 4 to 16 months of age. Sera from older children had the same action as those from adults. Forty-five adult sera killed 18 to 180,000 *Br. abortus* cells (strain 524) per ml. of serum (median 15000). Twenty-eight sera without agglutinins for Brucella from 14 individuals having dermal hypersensitivity for Brucella killed 18 to 18,000 Brucella (median 5000). The bactericidal power of 89 sera containing agglutinins from 26 patients having positive intradermal Brucella tests but no demonstrable bacteremia ranged from 0 to 1,800,000 (median 500). Forty-six sera from 13 patients having bacteremia due to *Br. abortus* killed only 0 to 180 organisms (median 36). The low bactericidal action of the latter group exhibited a pro-zone phenomenon in that the action was greatly increased by diluting out the sera and adding infants' sera for complement. This pro-zone phenomenon appears to be due to an excess of antibody. The killing action of normal sera having a high bactericidal titer is inhibited by the addition of sera from patients with bacteremia.

Pathogenesis of Pneumonia Secondary to Influenza. CARL G. HARFORD and (by invitation) HELEN V. LEIDLER, St. Louis, Mo.

Mice with sublethal influenza viral infection of the lung succumb more readily than normal mice to bacterial infection induced by inhalation of fine droplets containing pneumococci. A study of this phenomenon was designed to elucidate principles applicable to the general problems of respiratory infection.

Although it is known that marked multiplication of virus occurs in the lungs of mice within twenty-four hours after inoculation, microscopic lesions from the virus are not present at this early stage. On the other hand, histological lesions in the lung due to viral action are fully developed five days after inoculation.

Bacterial counts of the left lobes of the lungs of normal mice have shown a marked reduction in the number of pneumococci within six hours after inhalation and a similar reduction has been found in mice with twenty-four hour viral infections. In contrast, bacterial counts of the lungs of mice with five day viral lesions have demonstrated growth within six hours.

These observations indicate that the effect of the virus in lowering resistance to secondary bacterial infection is not dependent on the presence or multiplication of the virus in pulmonary tissue but is associated with the histological lesion produced by viral action.

The Pathogenesis of Circulatory Failure in Rocky Mountain Spotted Fever: Alterations in the Blood Volume and Thiocyanate Space at Various Stages of the Disease. GEORGE T. HARRELL and (by invitation) JERRY K. AIKAWA, Winston-Salem, N. C.

Previous investigation has shown that peripheral circulatory collapse in Rocky Mountain spotted fever is accompanied by a drop in serum proteins and the development of generalized edema.

Serial determinations of the blood volume and "extravascular thiocyanate space" were done at intervals during the course of the disease, and were compared with the

serum protein levels and with the intake and output of fluid. The blood volume was found to drop simultaneously with an increase in thiocyanate space; the magnitude of the alteration in the thiocyanate space was greater than that in the blood volume. These changes occurred between the tenth and the twelfth days of rash, and could be correlated with the clinical severity of the disease; a return to normal values took place early in convalescence, and was accompanied by diuresis.

The drop in serum proteins was preceded by an increase in urinary nitrogen excretion and could be partially alleviated by a very high-protein diet. Capillary permeability was altered in the disease, so that protein, electrolytes, and water escaped into the interstitial spaces. These changes occurred in 6 patients who received specific therapy in the form of hyperimmune antiserum or PABA, as well as in 7 patients who received supportive therapy only.

Similar alterations in blood volume and thiocyanate space have been produced experimentally in rabbits with serum sickness. An allergic mechanism may be responsible for the alteration of capillary permeability, although allergy in rickettsial diseases has not yet been demonstrated. Proper replacement of protein and the avoidance of excessive administration of crystalloids should help to prevent circulatory and respiratory failure. These principles of supportive therapy are probably applicable to other infectious diseases.

Penicillin Protection Against Bacterial Endotoxins. WALTER D. HAWK and ALDEN K. BOOR, Chicago, Ill. (introduced by C. Phillip Miller).

Penicillin provides a certain degree of protection for mice against intraperitoneal injections of the endotoxins of several gram negative bacteria.

The endotoxins against which a penicillin effect has been demonstrated have been prepared from meningococcus, gonococcus S. Paratyphi A., S. Paratyphi B., Shigella paradysenteriae, S. Enteriditis, S. Aertryeke and A. Aerogenes. Organisms are grown on solid media, harvested, washed in saline, lysed by dilute alkali (pH 8.0) sterilized by heat and finally cultured for proof of sterility. Graded doses of this endotoxin are given to mice intraperitoneally in order to determine its LD50 respectively for penicillin-treated and for untreated mice.

It is evident from a careful review of the data, that when penicillin is given in adequate amounts during the period preceding and shortly after endotoxin, a definite increase in LD50 follows.

Injection of control mice with the various materials used as vehicles for penicillin affords no protection.

Autopsy cultures of a representative number of mice have given no evidence of terminal infections to account for the higher mortality among controls.

Protection has evidently been provided, by means of penicillin administration against the endotoxins of all of the above named organisms. This result has not been apparent with the endotoxin of Shigella Shigae nor with several non-bacterial poisons tested.

The mechanism of this effect of penicillin cannot at present be explained.

Study of Folic Acid for Maintenance Therapy in Pernicious Anemia. ROBERT W. HEINLE and (by invitation) JANET T. DINGLE and AUSTIN S. WEISBERGER, Cleveland, Ohio.

Forty-one patients with pernicious or allied anemia were maintained on folic acid therapy for periods to one year. In 32 patients followed longer than six months, hematologic relapse occurred in nine who received intramuscular injections of 50 or 150 mg. at three- to six-week intervals. Relapse did not occur in any patient receiving daily oral doses of 10 to 40 mg.

Hematologic improvement occurred in six of the 41 patients. Three received daily oral therapy and three were given 45 or 50 mg. intramuscularly every two or four weeks.

Six patients in relapse responded adequately and have been maintained in good hematologic remission with folic acid.

Three of the 41 patients developed neurologic relapse while in hematologic remission. This complication was explosive and rapidly progressive in one. Liver extract therapy arrested the neurologic relapse in these patients.

It is concluded that daily oral therapy with ten or more mg. of folic acid maintained hematologic remission in these patients whereas intermittent intramuscular therapy permitted relapse in some cases. The occurrence of neurologic relapse indicates that folic acid is not a complete substitute for liver extracts, so that the latter must remain the treatment of choice for pernicious anemia.

Arterial Catheterization of the Left Ventricle, Left Auricle, and Pulmonary Vein. HARPER K. HELLEMS * (by invitation), LEWIS DEXTER and (by invitation), FLORENCE W. HAYNES, Boston, Mass.

Venous catheterization has made possible exploration of the right side of the heart and pulmonary artery, but has not been applicable to investigation of the dynamics of the left side of the heart and pulmonary vein. In the past, these chambers have been almost immune to investigation except by difficult and time-consuming procedures.

In the present study, a No. 6 French radiopaque catheter, with a curved tip as used for venous catheterization, has been introduced into the carotid artery of anesthetized dogs and guided fluoroscopically into the aorta, left ventricle, left auricle, and pulmonary vein. This may be accomplished without a change of respiratory rate, pulse rate, and arterial blood pressure. Although it is usually an innocuous procedure, damage to valves and endocardium has sometimes occurred. It has, therefore, not been deemed safe in patients.

In animals, changes in the pressure dynamics of the left side of the heart and pulmonary vein under conditions of cardiac stress will be discussed.

* Life Insurance Medical Research Fellow.

Changes in the Efficiency and Dynamics of Respiration in Experimental Congestion of the Lungs. HOWARD E. HEYER, JAMES HOLMAN and GEORGE T. SHIRES,* Dallas Texas (Introduced by Ben Friedman).

Experimental pulmonary congestion was produced in dogs by rapid venous infusion of 0.9 per cent solution of sodium chloride or by sodium chloride-sodium bicarbonate buffer solutions. Intrapleural pressure fluctuations increased markedly in total amplitude while mean intrapleural pressure rose toward atmospheric levels, and intrapleural pressure during expiration rose above atmospheric in every animal. Respiratory efficiency was determined by the tidal exchange produced for each centimeter change in intrapleural pressure. Efficiency of respiration and tidal exchange fell progressively with venous infusion, and rose rapidly when hyperpnea was induced by carbon dioxide inhalation. The changes accompanying pulmonary congestion were constant and were independent of alterations in hydrogen ion concentration or serum CO_2 tension. Vagotomy prevented acceleration of breathing during venous infusion but did not prevent the marked decrease in respiratory efficiency. Active participation of expiratory muscles was necessary to achieve optimal tidal exchange in the congested lung, since abolition of expiratory movements by lower cervical cord section caused marked diminution in tidal exchange. These findings were interpreted as being due to a marked increase in rigidity of the congested lung, so that tidal exchange was accomplished at the expense of a marked increase in activity of respiratory muscles, with a total greater change in intrapleural pressure. During extreme pulmonary congestion, even though intrapleural fluctuations increased markedly, tidal air diminished sharply, suggesting that (in addition to rigidity of the lung) alveolar space was encroached upon by the congested capillaries, or that the alveolar space was diminished in size by transudation of edema fluid.

Serum Sickness Due to Crystallized Bovine Albumin. JAMES T. HEYL (by invitation) and CHARLES A. JANEWAY,† Boston, Mass.

During experiments to test the possible value of albumin crystallized from bovine plasma by the method of Cohn and Hughes as a possible blood substitute, there was an opportunity to observe a severe and rather unusual form of serum disease in twenty-four patients. An intravenous dose of 25 grams of albumin was given to a group of 60 volunteers. Twenty-four of these developed symptoms. The striking clinical features of the disease were as follows: (1) long incubation period (10 to 33 days); (2) variation in severity from a very mild illness to a protracted one (4 to 6 weeks) with high fever; (3) the frequency of purpura, signs of hepatic and renal damage, and cardiac symptoms with electrocardiographic changes. Death occurred in one instance, and necrosis of the myocardium was found.

Immunological studies in the whole group indicated that bovine serum albumin, in man, as in experimental animals, is a relatively poor antigen in terms of its capacity to induce the formation of antibodies detectable by either the skin test or the precipitin test. In normal humans, as in animals, bovine albumin disappears very slowly from the circulation, whereas in certain instances of serum disease its disappearance was greatly accelerated. In some cases of severe serum sickness, recovery occurred before the disappearance of the bovine albumin from the circulation and antibodies never appeared. The relationship of the symptoms in the patients to the immunological phenomena will be discussed, as this experience with a disease caused by a measurable foreign protein without the power of multiplication raises many interesting questions concerning the pathogenesis of infactious diseases due to living agents.

Effect of Anxiety on the Cardiac Output. JOHN B. HICKAM, and WALTER H. CARGILL, Atlanta, Ga., (Introduced by Julian Ruffin).

Anxiety causes a marked increase in cardiac output. Twenty-three unselected medical students were studied immediately before and 24 hours after an important academic examination. As measured by the ballistocardiograph, the group showed an increase in cardiac output of approximately 50 per cent over the composed level. The greastest increase was 180 per cent.

Studies with the technique of intracardiac catheterization measuring the cardiac output by the Fick Principle were carried out on hospital patients. Anxiety was spontaneous or induced by suggestions; relative composure was obtained by reassurance, with or without light sedation. The data demonstrated that anxious individuals at rest may maintain a cardiac output which is much in excess of composed individuals, but without a corresponding increase in oxygen consumption. If an anxious person does sufficient work to increase the oxygen consumption to a level of 250 to 400 cc. per minute per square meter, the cardiac output may not be increased . Indeed, in certain subjects light work may cause the output to fall below that present in the resting but anxious state.

Studies on patients with left ventricular failure demonstrate that stimulus (work) which normally increases the cardiac output does not have this effect in severe heart failure. Instead the pulmonary pressure rises, presumably because of damming up of blood behind the failing left ventricle.

Reflex stimuli normally resulting in an increase in cardiac output would be expected to cause in patients with left ventricular failure a rise in pulmonary pressure. This offers a rational explanation for the precipitation of attacks of acute pulmonary edema by emotional stimuli and by reflex stimuli from the pleura or peritoneum during the removal of fluid by paracentesis.

* Done during the tenure of a Life Insurance Medical Research Student Fellowship.

† This work was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.

Streptomycin; an Antibiotic Effective Against Some Forms of Clinical Tuberculosis. H. C. HINSHAW and (by invitation) W. H. FELDMAN, Rochester, Minn.

The present status of streptomycin therapy in experimental tuberculosis of guinea pigs under varying conditions is briefly described and evidence presented to indicate that this drug is a highly effective antibacterial agent, even when utilized under unfavorable experimental conditions. The clinical possibilities and limitations of streptomycin for treatment are discussed briefly. Evaluation is based on experience with treatment of approximately 125 patients with different types of tuberculosis during the previous two and a half years. Clearly recognizable therapeutic effects are noted in many types of the disease, including tuberculous meningitis; miliary tuberculosis; progressive, exudative pulmonary tuberculosis; ulcerating tuberculous lesions of the larynx, trachea and larger bronchi; tuberculous draining sinuses, and tuberculosis of the genito-urinary tract. The effect of streptomycin appears to be limited to a suppression of the infectious process. The permanence of results may depend on whether or not natural healing may be achieved during the few months of effective action prior to the appearance of drug-resistant strains of Mycobacterium tuberculosis.

Extent of Vasodilatation Induced in Different Vascular Beds After Systemic Autonomic Blockade with Tetraethylammonium. S. W. HOOBLER, ROSALIE B. NELIGH, GORDON K. MOE, S. DON MALTON, SAUL COHEN, and H. T. BALLANTINE, JR., (by invitation) and R. H. LYONS, Ann Arbor, Mich.

In normotensive and hypertensive subjects injection of tetraethylammonium results in a temporary decrease in the total peripheral resistance as manifested by a reduction in mean arterial pressure and a maintained or increased cardiac output. The blood flow through vascular beds believed to be normally under some degree of vasomotor tone is markedly increased. The volume flow of blood in the hands and feet as measured by the venous occlusion plethysmograph increases significantly above resting levels following the administration of 500 mg. of the drug and exceeds the levels achieved by other vasodilating drugs and may occur despite a marked decrease in arterial pressure. Blood flow seldom reaches the levels seen after sympathetic block with metycaine or after heat to the trunk, suggesting that a complete autonomic blockade is not produced. On the other hand there is a rise in cutaneous circulation as judged by increase in skin temperature of the hands and feet frequently with abolition of the temperature gradient. After sympathetic denervation of the extremity the blood flow is not further increased by administration of this drug. Induced vasoconstrictor reflexes in the hands and feet are reduced or abolished after tetraethylammonium. Blood flow in the forearm is only slightly increased after administration of the drug. Renal blood flow as measured by para-amino hippurate clearance is unaffected despite variable reductions in arterial pressure in hypertensive and normal subjects. It is suggested that increase in blood flow through various vascular beds following tetraethylammonium depends on the presence of neurogenic vasoconstrictor tone in these areas.

Sickle Cell Anemia. I. The Structural Defect of the Erythrocytes in Sicklemia. WILLIAM H. KELLY and (by invitation) P. K. SWITZER, GEORGE MANGAN, WILLIAM M. MCCORD and B. F. CULP, Charleston, S. C.

Preliminary studies are in process of completion to show that the error of construction of erythrocytes in sicklemia resides in the cell membrane or stroma.

The erythrocytes in sicklemia exhibit an increased hematocrit value when reduced, whereas, with normal red cells the reading is equal or tends to be reduced under the same conditions.

An analysis of samples shows the increase in the hematocrit value of reduced sicklemic erythrocytes is attended by at least a proportionately greater amount of intercellular fluid. This indicates that the reduced red cells of sicklemic blood are not of increased size but possess a greater viscosity than oxidized normal or sicklemic erythrocytes.

A comparison of the cell shadows from laked sicklemic and visual erythrocytes by the above technic showed apparently identical differences.

Cerebral Blood Flow and Metabolism in Diabetic Acidosis and Coma. SEYMOUR S. KETY, B. DAVID POLIS, CARL S. NADLER and CARL F. SCHMIDT, Philadelphia, Pa. (Introduced by Isaac Starr).

Quantitative measurement of cerebral blood flow and metabolism of 13 patients in profound diabetic acidosis or coma was made by means of the nitrous oxide method. On admission the mean values for blood glucose, arterial CO_2 content, and arterial pH were 560 mg. per 100 cc., 12 volumes per cent and 7.06 respectively. No significant differences with respect to age, blood pH, CO_2, glucose electrolyte patterns or cerebral blood flow were demonstrable between patients who recovered and those who succumbed. Those who recovered exhibited higher cerebral oxygen consumption, better mental function, higher blood pressure and lower blood ketone concentration. A reduction in cerebral oxygen consumption of 40 per cent from the normal was associated with failure to respond to treatment and death within 12 hours. These data, hitherto unobtainable, permit an evaluation of numerous factors held to play a role in the cerebral depression and frequent irreversibility of this condition. Correlation of cerebral oxygen consumption with some of these functions reveals the following: poor correlation with blood glucose, CO_2, total base or chloride concentrations, or with cerebral blood flow; a just significant correlation with arterial hydrogen ion activity ($r = 0.43 \pm \sigma_r\ 0.187$); the best correlation was obtained with blood ketone concentration ($r = -0.65 \pm \sigma_r\ 0.132$).

The Rate of Conduction in the Human Atrium. C. E. KOSSMANN, and (by invitation) A. R. BERGER, J. BRUMLIK and S. BRILLER, New York, N. Y.

The classical experiments of Lewis and his associates in which they demonstrated that the rate of electrical conduction in the dog's atrium is 1,000 mm. per second were repeated, with modifications, in man.

By means of two string galvanometers and an electrode passed into the right atrium through an antecubital vein,

intraatrial potentials at various levels between the cavae were recorded simultaneously with a standard lead. Distances between levels were measured radiographically by two techniques. Times of intrinsic deflections were measured using a fixed wave in the simultaneous lead as a reference point. In three men with normal sized hearts the calculated rates of conduction in the right atrium were 2,275 mm. 2,666 mm. and 1,866 mm. per second respectively.

Assuming that the spread of excitation through the atria is uniform and knowing that the mean duration of the P wave in the human electrocardiogram is 0.08 second, the distance from the region of the sino-auricular node to the farthest reaches of the atria, most probably the tip of the left atrial appendage, is calculated to be approximately 18.5 cm. Measurements on models, on cross-sectional anatomical drawings, and on fresh human hearts at necropsy indicate that the distance in question is approximately 15 to 22 cm., and that the anatomical considerations are in accord with the physiological observations.

The conclusion reached is that in a sinoatrial rhythm electrical excitation travels two or more times faster in the human than in the canine atrium.

Calcinosis, Scleroderma and the Anti-Stiffness Factor. John Lansbury, Rosalind Wulzen, Lawrence W. Smith and William J. van Wagtendonk, Philadelphia, Pa. (Introduced by Richard A. Kern).

The anti-stiffness factor was detected by Wulzen and Bahrs in greens and isolated from raw cream and cane juice by van Wagtendonk and Wulzen. Guinea-pigs lacking it develop muscular stiffness and degeneration and eventually deposits of calcium between muscle fasciculi, around joints, under the skin and in parenchymal organs. The anti-stiffness factor reverses the early changes. It appears to be a regulator of phosphorus metabolism.

This communication describes the roentgen appearance of the calcifications and compares them with the deposits found in human calcinosis. Striking similarities to both circumscribed and interstitial types are noted. (These are associated with scleroderma and dermatomyositis in humans.) The lesions induced by excess vitamin D are compared and excluded as the cause of both animal and human lesions.

Since the lesions induced at will in guinea-pigs by deficiency in the anti-stiffness factor are roentgenographically identical with those of human calcinosis, we postulate that a deficiency of this or similar substances may be one of the factors in the pathogenesis of human calcinosis and possibly scleroderma and dermatomyositis.

The Effects of Radiation on Hemopoiesis. Is there an Indirect Effect? John S. Lawrence and (by invitation) William N. Valentine and Andrew H. Dowdy, Rochester, N. Y.

Twenty-six successful cross circulation experiments (carotid-to-carotid anastomoses) were performed between a normal cat and one which had received 1500r whole body radiation at some interval up to eighty-two hours before cross circulation. In seven experiments, one animal was radiated at the time the carotid anastomoses were functioning, the normal partner being shielded during radiation. The total and differential leukocyte counts of the normal cat were followed for approximately twenty-eight days after cross circulation was discontinued.

The data were not considered to support the thesis of indirect effects *peculiar* to radiation mediated via the circulation. A trend toward slightly lowered absolute lymphocyte counts in normal animals after cross circulation was not considered significant and in no instance did leukopenia develop in the normal animal. A review of the medical literature revealed no convincing support for indirect radiation effects.

The clinical impression that leukopenia results in humans given repeated radiation either over non-hemopoietic tissue or over very limited amounts of hemopoietic tissue needs to be very critically reviewed.

Clinical Hypoprothrombinemia: A Study of Factors A and B of Prothrombin. Jessica H. Lewis and Ivan L. Bennett, Jr., Atlanta, Ga. (Introduced by Eugene A. Stead, Jr.).

Clinically, hypoprothrombinemia may be due to one or more of the following causes: (1) vitamin K deficiency, due to poor diet or poor absorption, (2) severe liver damage, (3) administration of drugs (dicoumarol and salicylates), and (4) idiopathic or unexplained hypoprothrombinemia.

Quick (1943) and Feissly (1943) have shown that the prothrombin reaction is dependent upon the presence of at least two substances in normal plasma. Factor A is reduced by aging or heating plasma and Factor B is reduced in vivo by administration of dicoumarol or in vitro by absorption with aluminum hydroxide or barium sulfate. Standard methods of preparing Factor A and Factor B plasma are presented and attempts are made to characterize these factors by the effects of heat, pH, and Ca^{++} ion on each. Employing preparations of these factors, studies were made on plasma obtained from patients with the various types of prothrombin deficiencies.

As examples of each type of prothrombin deficiency patients manifesting symptoms of obstructive jaundice, Laennec's cirrhosis, dicoumarolization, and idiopathic hypoprothrombinemia are presented.

Our patient suffering from idiopathic hypoprothrombinemia is unique in our experience and among the rare published cases. The onset was abrupt at age 29 with almost complete disappearance of Factor B of prothrombin, the Quick prothrombin time being prolonged to 10 minutes with a whole blood coagulation time prolonged to 60 minutes. Fibrinogen and formed elements of the blood were normal. The patient received massive dosage of vitamin K, with simultaneous return of circulating prothrombin. Under observation she has maintained normal prothrombin without treatment for 60 days.

In all four types of prothrombin deficiency there was demonstrable a similar defect, that is, reduction of Factor B with apparently normal Factor A. Administration of vitamin K either as synthetic 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester tetrasodium salt

(synkavite) or 2-methyl-3-phytyl-1,4-naphthoquinone (vitamin K-1) caused rapid regeneration of Factor B unless severe liver damage was present.

These investigations suggest that vitamin K is necessary for the formation of Factor B of prothrombin. Since no clinically demonstrable deficiency of Factor A was found in these patients, the role of vitamin K in relation to this factor is problematic.

The Direct Isolation of Mumps Virus in Chick Embryos. GLEN R. LEYMASTER and THOMAS G. WARD, Baltimore, Md. (Introduced by Thomas B. Turner).

Successful direct isolation of mumps virus has not been reported in this country, so far as the authors are aware. Mumps virus has been isolated in this laboratory by chick embryo inoculation, from 4 of 5 patients ill with parotitis during the first four days of illness. Two of these strains have been inoculated into monkeys, which developed clinical and serological evidence of mumps. Each strain was re-isolated from monkey parotid by embryo inoculation.

Penicillin and streptomycin are added to the patient's saliva to a final concentration of 250 and 2500 units per milliliter, respectively. One-tenth milliliter of the saliva-antibiotic mixture is then inoculated into the amniotic sac of seven day old embryos.

After seven days incubation at 35° C., amniotic fluid and membranes are removed. The fluid is tested by chick cell hemagglutination. Positive fluids are re-inoculated. The amniotic membranes of negative specimens are ground with their respective amniotic fluids, and re-inoculated. Three passages are carried out before a specimen is considered negative.

Identification of the virus as mumps is accomplished by hemagglutination inhibition and complement fixation tests, using amniotic fluid as antigen.

Further laboratory studies, including neutralization tests in embryos, are now in progress.

Fluorometric Measurement of Plasma Quinidine and its Correlation with Cardiac Effects in Man. ARTHUR J. LINENTHAL, STANLEY ULICK and LAWRENCE A. PATTERSON, Boston, Mass. (Introduced by Joseph E. F. Riseman).

Measurement of the fluorescence of quinidine provides a simpler and more sensitive means of determining its concentration than has previously been available.

In man, within fifteen minutes after a single oral dose, quinidine can be detected in the plasma. Maximum levels are reached in 1 to 3 hours and may be maintained for 2 to 3 hours. Appreciable amounts remain after 8 to 12 hours. Average peak concentrations after single doses of 0.2, 0.4 and 0.6 gm. are, respectively, 0.8, 1.3 and 2.0 mg. per liter.

When the same dose is repeated every two hours, *i.e.* at the peak response to the preceding dose, the concentration increases with the first four or five doses only. A further increase in concentration can be attained more effectively by increasing the size of the individual dose than by increasing the frequency of administration.

When the same dose is repeated every 6, 8 or 12 hours, peak concentrations are similar and do not increase. With the more frequent administration the level is better maintained between doses.

Suitable plasma concentrations for the therapy or prophylaxis of cardiac arrhythmias can thus be attained by planned doses according to one of the general schedules described above.

Plasma concentrations parallel closely the effect on the heart as measured by the rate of oscillations in auricular fibrillation, and by the Q-T duration. This correlation affords a new quantitative approach applicable to the clinical and pharmacological study of this cardiac drug.

The Application of the Isotope Technique to the Study of the Rates of Formation of Blood Constituents in Man. IRVING M. LONDON, DAVID SHEMIN, and D. RITTENBERG, New York, N. Y. (Introduced by R. West).

The feeding of glycine labeled with N^{15} to humans results in the incorporation of N^{15} in the serum proteins and the heme of red blood cells as well as in other body constituents. Glycine is the nitrogenous precursor of the protoporphyrin of hemoglobin. The N^{15} is incorporated in the red blood cell during the deposition of hemoglobin, and the labeled porphyrin remains in the cell until the cell disintegrates. The porphyrin is not subsequently reutilized for hemoglobin synthesis. Accordingly, the isotope technique affords a unique method for studying the kinetics of red cell formation and for determining the average life span of the human red cell. This life span has been found to be 127 and 128 days in two normal adult men. Data on the rates of generation and on the life span of red cells in a patient with pernicious anemia and in another with polycythemia vera have been obtained which may clarify certain factors in these disease processes. Serial measurements of N^{15} concentration in electrophoretically separated fractions of serum proteins have yielded information on the relative rates of elaboration of these proteins. Correlative studies of N^{15} concentration in urinary urea, ammonia and uric acid have provided further information on protein and purine metabolism.

The Relation of the Rh Factor in Cirrhosis of the Liver. FRANK S. LOVINGOOD, Philadelphia, Pa. (Introduced by Franklin R. Miller).

Of the various factors listed as contributing to the production of cirrhosis of the liver, erythroblastosis fetalis and the familial tendency are probably mentioned least often. One is able to find several reports of each type in the literature.

Recently twenty cases of cirrhosis of the liver have been studied; 45 per cent or nine patients were found to be Rh negative and 55 per cent or eleven patients were Rh positive. The percentage of Rh negative patients seems significant.

Cirrhosis that follows icterus gravis neonatorum is probably due to an antigen antibody reaction. The mother forms antibodies against the Rh agglutinogen of the fetus. The antibody passes into the fetal circulation resulting in hepatic damage.

The development of the Rh negative and familial cirrhosis may be identical in that the development may be

due to incompatibility between a Rh positive mother and a Rh negative fetus. Infants born under such circumstances, as well as those born with icterus gravis neonatorum, probably begin life with decreased hepatic reserve and, therefore, are more prone to develop clinical cirrhosis of the liver later in life. The time of development will depend on the degree of initial damage, as well as the degree of further damage from infection, ingestion of alcohol or poor dietary measures.

A Study of the Mechanism of Nephrotic Edema. JOHN A. LUETSCHER, JR., Baltimore, Md.

The association of hypoproteinemia, sodium retention, and edema in nephrosis is well known, but the fundamental mechanisms leading to these manifestations are obscure. The decreased colloid osmotic activity of the plasma may facilitate the development of edema, but is not necessarily related to the presence or absence of clinical edema.

The repeated administration of concentrated human serum albumin to patients with nephrosis increases the concentration of albumin and the colloid osmotic activity of plasma. The consequent large shift of fluid into the plasma minimizes the rise in protein concentration despite the greatly increased circulating protein. These impressive circulatory changes are followed by smaller and sometimes ineffectual changes in the renal excretion of salt and water. The glomerular filtration rate is generally increased, and the proportion of sodium reabsorbed is greatly diminished. The moderate diuresis of sodium is not necessarily correlated with increased urine flow or with excessive proteinuria, and is not reproduced by the administration of mannitol, an osmotically active and unreabsorbed substance.

Spontaneous diuresis in nephrosis is distinctively different from the diuresis induced by the administration of albumin in that the plasma volume and protein concentration may change only slightly during a spontaneous diuresis, while the proportion of sodium reabsorbed by the renal tubule is diminished even more than after albumin therapy.

It is concluded that the reabsorption of an excessive proportion of sodium by the renal tubule is responsible for the intractable edema of nephrosis, and that the restoration of the total circulating protein to nearly normal levels does not immediately lead to diuresis. The relationships between hypoproteinemia, edema, and renal function are being further investigated.

The Pharmacologic Promotion of Evacuation from the Post-Vagotomy Retentive Stomach. THOMAS E. MACHELLA and (by invitation) HORACE H. HODGES and STANLEY H. LORBER, Philadelphia, Pa.

One of the complications which had developed following section of the vagus nerves for peptic ulcer has been gastric retention. This has occurred especially in those patients who have not had a gastroenterostomy or who do not have an adequately functioning one. For the relief of retention, additional surgery, some form of gastroenterostomy, has had to be performed.

It has been found possible to avoid surgery in each of six patients with post-vagotomy gastric retention by promoting gastric evacuation by means of the parasympathomimetic drug, urethane of B-methyl choline (Urecholine).

The drug is administered orally after each of the main meals of the day in dosage of 6 to 10 mg. The patients have remained free of symptoms of retention while taking the drug but not when a "placebo" was substituted. Within 5 to 10 minutes of a subcutaneous injection of a 5 to 10 mg. dose, peristaltic activity in the antrum of the stomach can be demonstrated roentgenologically or by means of a recording balloon. The drug does not give rise to a significant increase in free hydrochloric acid providing the patient is permitted to swallow saliva or if the stomach contains neutralizing food substances.

Osmotic Factors Influencing the Formation of Ascites in Patients with Laennec's Cirrhosis of the liver. HAROLD MANKIN and ALICE LOWELL, New York, N. Y. (Introduced by Arthur J. Patek, Jr.).

Effects of experimental alteration of the colloid osmotic pressure of the plasma and ascitic fluid and of the intraperitoneal hydrostatic pressure were studied in 10 patients with cirrhosis and ascites. The colloid osmotic pressures of the plasma and ascitic fluid were measured directly in an Hepp osmometer. The ascitic fluid volume was measured by an adaptation of the Evans Blue dye technique.

In each patient the difference between the colloid osmotic pressures of the plasma and the ascitic fluid remained approximately constant through periods of 3 months to 1 year. Following (1) alteration of the intraperitoneal hydrostatic pressure by abdominal paracentesis, (2) increase of the colloid osmotic pressure of the plasma during mercurial diuresis, (3) decrease of the colloid osmotic pressure of the plasma after the oral administration of salt solution, or (4) decrease of the colloid osmotic pressure of the ascitic fluid by the intraperitoneal injection of physiological saline solution, there were temporary changes in the rates of fluid and protein exchange between the plasma and ascitic fluid which served to restore the pre-existing constant difference between the colloid osmotic pressures of the plasma and ascitic fluid.

The results are interpreted as consistent with the Starling hypothesis describing osmotic equilibrium between transudates and the plasma.

A Study of the Plasma and Tissue Globulins in Myelomatosis. NICHOLAS H. MARTIN, Boston, Mass. (Introduced by James L. Gamble).

In a patient with multiple myeloma, the total plasma proteins were estimated as 10.9 grams per 100 mls. plasma. By electrophoretic analysis, 65 per cent of the total moved with the mobility of γ-globulins. There was no apparent excess of α or β globulins. This γ-globulin was separated from the other plasma proteins and compared with γ-globulin from normal human plasma. It moved as a discrete well-defined entity having a mobility of the order of 1.1×10^{-4} cm/volts/sec. Nevertheless it

was heterogeneous in the ultracentrifuge, having a pattern quite unlike that of γ-globulins obtained from normal plasma. Moreover, it precipitated from an acetate buffer, ionic strength, 0.01 mols/L and of pH 6.0. In this also it differed from γ-globulin from normal human plasma.

The patient died in hospital, and a diagnosis of multiple myeloma was confirmed at autopsy. Tumor tissue was taken at autopsy, frozen, washed free of blood, and saline preparations examined. Ultracentrifugal studies showed the presence of abnormal globulins similar to those identified in the plasma. Similar globulins, though in much smaller amounts, were identified in the splenic pulp. Examination of control tissues from a normal individual treated in the same way, gave no indication of the presence of these globulins.

It is suggested that the γ-globulin circulating in the plasma of patients suffering from myelomatosis is abnormal and arises from the tumor cells.

Streptomycin in the Treatment of Tuberculosis in Humans. WALSH McDERMOTT, CARL MUSCHENHEIM, and SUSAN HADLEY, New York, N. Y. (introduced by David P. Barr).

Since January, 1946, a clinical and laboratory investigation of the treatment of tuberculosis in humans has been conducted. More than fifty patients have been treated with a uniform preparation of streptomycin sulfate. The drug was prepared from crystalline material and is believed to be at least 95 per cent pure. The experimental series includes fourteen patients with acute hematogenous tuberculosis or meningitis and approximately forty individuals with pulmonary disease. The majority of the latter infections were either caseo-pneumonic or predominantly exudative in type. Approximately two-thirds of the series have been observed for periods of six to twelve months after the institution of the antibacterial therapy.

The course of the infection before, during and after streptomycin therapy has been studied by frequent clinical, roentgenological and bacteriological observations. Histologic studies of the nature of the tuberculous lesions have been made on the tissues of five individuals who had received streptomycin for periods ranging between 90 and 300 days before death.

The in vitro streptomycin sensitivity of tubercle bacilli isolated from each patient at biweekly intervals has been determined in Dubos media. The development of in vitro drug resistance appeared after four to eight weeks of therapy in the majority of patients from whom tubercle bacilli could still be cultured at that time. A definite correlation was found between the streptomycin sensitivity of a patient's organism in vitro and the response of that particular infection to the continued administration of the drug.

The institution of streptomycin therapy was associated, in every instance, with a definite and sometimes dramatic effect upon the course of the tuberculous infection. In certain forms of the disease, notably in acute hematogenous tuberculosis, a high incidence of relapse resulted from the development of drug-resistant strains of tubercle bacilli. The most uniformly satisfactory results were observed in the treatment of early exudative infections, moderately or far advanced in extent and which were usually associated with thin-walled cavities.

"Forward Failure": The Mechanism of Cardiac Edema Formation in Subjects with Normal or High Cardiac Outputs. ARTHUR J. MERRILL and WALTER H. CARGILL, Atlanta, Ga. (Introduced by Paul B. Beeson).

Previously presented evidence favoring the forward failure concept of edema formation in advanced heart failure consists of the following: (1) Marked reduction in renal plasma flow and filtration rate which is independent of venous pressure levels and closely related to inadequacy of cardiac output. (2) Filtration rates below approximately 70 cc. per min. result in a critical reduction of the amount of salt and water presented to the tubules. (3) Almost complete reabsorption by the tubules occurs with a net retention of salt and water and consequent edema formation. The cause of edema in patients with low cardiac reserve, but an adequate resting cardiac output and renal function, has not been explained; nor has edema associated with a high cardiac output, as in thyrotoxic heart disease.

The present work deals with a study of the effect of light exercise on the renal blood flow of patients in whom light exercise would be expected to bring out inadequacy of cardiac reserve. It was necessary that they have a high enough renal plasma flow and filtration rate to make possible a demonstration of significant reduction with exercise. Seven of 10 cardiac subjects had a fall in filtration rate with exercise which approached the critical level of 70 cc. per min. Of 9 normal individuals, none had an appreciable fall in filtration rate, though a few showed a substantial decrease in renal plasma flow.

In thyrotoxicosis the cardiac output parallels the BMR. Eight thyrocardiac patients with mild and moderate failure showed no striking change in renal plasma flow and filtration rate. One with severe failure in the presence of a high cardiac output had marked diminution in renal blood flow and filtration rate; these rose to normal as the BMR fell. One other patient with severe failure had a low renal blood flow but the filtration rate was normal.

Thus it appears that while there is no fixed level of cardiac output at which the renal plasma flow and filtration rate fall, reductions may occur when the tissue demands exceed the ability of the heart to respond. If the filtration rate becomes sufficiently low, retention of salt and water (edema) occurs.

Possible mechanisms of the renal shutdown are mentioned.

*Renal Blood Flow and Sodium Excretion Studies in Congestive Heart Failure.** REUBEN MOKOTOFF and GEORGE ROSS, New York, N. Y. (Introduced by Louis Leiter).

A study of the renal plasma flow, glomerular filtration rate, and sodium excretion in congestive heart failure was

* Aided by a grant from the Martha M. Hall Foundation and the Committee on Scientific Research of the American Medical Association.

undertaken, since it has been postulated that cardiac edema is the result of failure of the kidney to excrete sufficient amounts of sodium and water. We found, in a series of 16 cases of chronic congestive heart failure due predominantly to rheumatic heart disease, that the mean "effective" renal plasma flow was 30 per cent and the mean glomerular filtration rate 65 per cent of normal. The mean filtration fraction was increased to 36.7 per cent as compared to 16.6 ± 1.5 per cent in our group of 14 normals. Five rheumatic cardiacs not in heart failure had normal glomerular filtration rates, renal blood flow and sodium clearances.

In 2 congestive failure cases in which 4 per cent sodium chloride was given, there was an excretion rate of 1 per cent and 1.5 per cent during the test period as compared to 7.4 per cent and 9 per cent in two normal patients under similar test conditions. The decreased sodium excretion found in congestive heart failure is due to a diminished filtration rate and not to an increased tubular reabsorption since the rate of reabsorption is the same for the cardiac in failure and for the normal and is a direct function of the glomerular filtration rate. It will be shown that the following equation expresses, as a first approximation, the relationship between the rate of tubular reabsorption (T) and the glomerular filtration rate (C_m):

$$T = P_{Na} C_m$$

where P_{Na} Equals the plasma concentration of sodium and represents the slope of the line. Physiologically induced variations in the filtration rate from 100 to 219 cc. in the normal and from 60 to 90 cc. in the cardiac also reveals constancy in the rate of sodium reabsorption. This amounts to a mean of 13.3 millimols per 100 cc. of glomerular filtrate under our experimental conditions.

Our findings are consistent with the views as outlined by Stead and Merrill that in chronic congestive heart failure there is a decreased sodium load presented to the tubules for reabsorption, which fact is responsible for the series of events—increased blood volume, increased venous pressure—leading to cardiac edema.

Use of Chemotherapeutic and Antibiotic Agents in the Isolation of Viruses from Contaminated Sources. HERBERT R. MORGAN, Boston, Mass. (Introduced by Maxwell Finland).

This is part of a study designed to test the usefulness of certain chemotherapeutic and antibiotic agents as aids in the direct isolation of viruses in eggs from contaminated sources such as sputum, urine and feces. Psittacosis virus was used since it occurs in the nasal secretions and feces of its natural host. A standard preparation of seed virus was used throughout the study and varying dilutions of the virus and different amounts of the test agents were injected into the eggs by the yolk sac route.

Sulfadiazine and penicillin were found to inhibit the growth of the virus while streptomycin and tyrothrycin had no appreciable effect. Streptomycin alone and in combination with tyrothrycin was found useful for the isolation of psittacosis virus from fecal material on direct injection of eggs via the yolk sac route, both with virus added to normal human feces and from the feces of mice infected with psittacosis virus by the intravenous route. P-aminobenzoic acid and folic acid were found to counteract the inhibitory action of sulfadiazine, but had no effect on the multiplication of the virus. Cysteine HCl itself had no effect on the virus. When injected as long as 4 hours after the penicillin and virus, cysteine HCl inhibited the action of the penicillin on the growth of the virus.

A Study of Forty Patients Who Died in Liver Coma. T. L. MURPHY, T. C. CHALMERS and R. D. ECKHARDT, Boston, Mass. (Introduced by George R. Minot).

A study is presented of 40 autopsied patients who died in coma within the past two years, all of whom had severe primary liver disease. The coma was precipitated by infection in seventeen, and hemorrhage in three. No extra-hepatic factor was found in the remaining twenty ("pure coma").

The clinical and liver function studies of the patients in "pure coma" did not differ significantly from their pre-coma studies nor from those of other patients with severe liver disease not in coma. The concentrations of blood non-protein nitrogen, glucose, chloride, carbon dioxide combining power, bilirubin, and phenols, although frequently abnormal, were not sufficiently altered to account for the coma. Determinations of plasma alpha amino nitrogen in comatose patients were normal.

Confusion and delirium for which sedatives were used often preceded the coma, and in six of the uncomplicated cases such sedation induced deep sleep which merged imperceptibly into coma.

Except in two patients in whom concentrated glucose temporarily lightened coma, the administration of intravenous dextrose, vitamin B complex, liver extract, protein hydrolysate and prophylactic chemotherapy failed to alter the coma. In the past two years there have been no patients with severe liver disease alone who recovered from coma. After prolonged treatment two have recovered from coma secondary to sedation. Two patients with infection which induced coma recovered after similar parenteral nutrition and control of infection.

It is concluded that although clinical and laboratory examinations do not elucidate the etiology of liver coma, sedation as well as infection and hemorrhage is an important contributing factor. Intravenous glucose, B complex vitamins, liver extract and protein hydrolysates are therapeutically supportive but are not specific analeptics.

Correlation of Electrocardiographic and Pathologic Findings in Infarction of the Lateral Wall of the Left Ventricle. GORDON B. MYERS and (by invitation) HOWARD A. KLEIN and BERT E. STOFER, Detroit, Mich.

Lateral infarction was established by necropsy in ninety cases. Pathologic examination included coronary injection with radio-opaque mass, section by Schlesinger technique or transverse slices, roentgenogram, and multiple microscopic blocks. Standard limb and Wilson precordial leads were available in every case and Goldberger extremity leads were obtained in eighty cases.

Anterior infarction extending into the lateral wall of the apex was found at necropsy in forty-six cases. Lateral extension was manifested by abnormal Q waves in $V_{5,6}$ and usually in aV_L, whereas infarction confined to the anterior wall was manifested by abnormal Q waves in $V_{2,3,4}$. Lateral extension from posterior infarction was found in twenty-five cases. A diagnosis of lateral extension could be made from V_6 or aV_L in eleven cases and of posterior infarction from aV_F in seventeen of twenty cases. Primary lateral infarction was present in nineteen cases. Infarcts confined to the lateral aspect of the apex were recognizable from the pattern in $V_{5,6}$ and aV_L, whereas those localized to the lateral aspect of the base could usually be suspected from aV_L and confirmed by high precordial leads.

The infarct was generally maximal in or limited to the subendocardial layer and the thickness could be estimated from depth and duration of the Q wave. Infarcts confined to the subepicardial layer were accompanied by abnormalities in the T, but not in the Q wave.

The correlation of electrocardiographic and autopsy findings will be brought out by the presentation of illustrative cases.

Measurement of the Hepatic Blood Flow in Man. J. D. MYERS, Atlanta, Ga. (Introduced by W. M. Nicholson).

Measurement of the hepatic blood flow in normal males has been undertaken utilizing a method based on "the Fick principle" with urea as the test substance. The urea concentration has been determined in (1) femoral arterial blood, which has been assumed to have the same urea concentration as hepatic arterial and portal venous blood, and (2) hepatic venous blood which has been obtained by direct catheterization of the right hepatic veins by the procedure of Warren and Brannon. The urea determinations have been done by a modification of the urease method of Van Slyke and Cullen with an accuracy of ± 0.2 mg. of urea per 100 cc. of blood. Urine has been quantitatively collected and assayed for urea by the same procedure.

In fasting, normal, young males, the arterial urea concentration has generally been constant over the period of study, *i.e.* about two hours. Accordingly, the urinary excretion of urea during this period is considered equal to the production of urea by the liver. The excretion of urea in the urine has varied between 12.1 and 16.4 mg. per minute, and the hepatic vein — femoral artery urea difference has ranged from 0.5 to 1.2 mg. of urea per 100 cc. of whole blood. The calculated hepatic blood flows per minute,

$$\frac{\text{Urinary excretion (hepatic production) of urea per minute}}{\text{Hepatic vein} - \text{Femoral artery urea difference}}$$

range from 750 to 1,620 cc. per square meter of body surface. Concomitant determinations of cardiac output have shown these hepatic blood flows to represent 19 to 45 per cent of the entire cardiac output.

Estimated splanchnic oxygen consumptions (femoral artery — hepatic vein oxygen difference × hepatic blood flow) vary between 27 and 77 cc. of oxygen per minute, which represents from 11 to 31 per cent of the total oxygen consumption at rest.

Comparison is made of the data for liver blood flow by the urea method with data obtained simultaneously by the bromsulphthalein method of Bradley *et al.*

The Improved Demonstration of Circulating Antibodies in Hemolytic Anemia by the Use of a Bovine Albumin Medium; Parallelism with Survival Time of Transfused Red Cells. JACOB NEBER, Boston, Mass. (Introduced by William Dameshek).

Circulating antibodies were investigated in the sera of seventeen patients with various hemolytic syndromes by a modification of the tube agglutination technique in which 20 per cent bovine albumin solution completely replaced normal saline as a diluting and suspending medium.

In five cases of idiopathic acquired hemolytic anemia a circulating warm antibody was demonstrated with the albumin technique, although in four of these cases, antibody could not be demonstrated in a saline medium. Three patients with congenital hemolytic anemia not in crisis showed no antibody, but in one of these patients during a hemolytic crisis a warm autohemolysin was demonstrated in bovine albumin only.

Two patients with sickle cell anemia, two with severe Mediterranean anemia, one with acute hemolytic anemia caused by chemical exposure, one with paroxysmal nocturnal hemoglobinuria, two patients with hypersplenic hemolytic anemia, and one patient with the symptomatic hemolytic anemia of subacute lymphatic leukemia failed to show circulating antibodies.

Parallel determinations of the survival time of transfused red cells by the Ashby technique showed a distinct correlation with the presence of antibodies. The survival time was greatly diminished when antibodies were demonstrable. Performance of both types of tests proved of distinct diagnostic and prognostic value.

Studies on the Control of Hepatitis Virus in Blood and Blood Products. JOHN R. NEEFE, and (by invitation) JOSEPH STOKES, JR., SYDNEY S. GELLIS, and MERCER A. BLANCHARD, Philadelphia, Pa

The transmission of hepatitis viruses by intentional or accidental parenteral introduction of blood or its products is a serious current problem and promises to be one of equal, or possibly greater, importance in the future. Its present magnitude is indicated by the fact that the chiefs of service in some leading hospitals will no longer permit the use of pooled plasma (including the large supplies of surplus Red Cross plasma) in their patients. The obvious need for the development of methods for inactivation and control of the hepatitis viruses in blood or its products has led to the preliminary studies summarized herein.

1. In a cooperative study conducted in conjunction with the Harvard Plasma Fractionation Group, a serum hepatitis virus in human serum albumin solution apparently was inactivated, without rendering the albumin solution unsatisfactory for human use, by heating the experimentally contaminated albumin solution for 10 hours at 60° C. after stabilizing it with acetyl tryptophane and sodium caprylate. As this method is compatible with large scale production, it appears that, until proved otherwise, human

albumin solutions so treated can be considered free of the risk of virus hepatitis and that this valuable blood product, at least, could be salvaged safely from the large stores of pooled plasma suspected or known to be contaminated with a hepatitis virus.

2. The demonstrated protective effect of gamma globulin against infectious hepatitis has led to investigation of its protective effect against serum hepatitis. No protective effect was apparent when 10 ml. of gamma globulin and 2 ml. of plasma (known to contain a serum hepatitis virus) were injected simultaneously at separate intramuscular sites. No neutralizing or protective effect was apparent when 2 ml. of the plasma were mixed with 1 ml. of gamma globulin and allowed to stand for 4 hours at room temperature before intramuscular injection of the mixture into volunteers. The results of the clinical trials in army hospitals have suggested that quantity and/or time of injection may be important factors in the protective effect of gamma globulin against serum hepatitis. As the same factors might account for the lack of apparent protective effect in the experimental study described, these factors have been taken into consideration in a subsequent study now nearing completion. Twenty-one volunteers have been injected with plasma known to contain a serum hepatitis virus. Thirteen to sixteen ml. of gamma globulin have been injected at monthly intervals into 7 of these men starting 1 month after the plasma injection. Another 7 men serve as controls for the first group injected with globulin and for a third group of 7 men each of whom was injected with a mixture of the plasma and gamma globulin (3 ml. plasma and 10 ml. of gamma globulin, mixed and stored for 3 hrs. at room temperature and for 24 hrs. at 4° C before injection). The results of this study will be presented and discussed together with the results of the studies outlined above.

The Effect of Coronamide in Inhibiting Penicillin Excretion. R. F. Parker and (by invitation) R. Albright, Cleveland, Ohio.

Penicillin injected intramuscularly or intravenously is rapidly excreted in the urine, largely as the result of renal tubular activity. A number of attempts have been made to increase the concentration of penicillin in the blood, and to prolong the effect of a given dose by suppressing the tubular secretion of the drug. Coronamide (4 carboxyphenyl-methane sulfonanilide) has recently been proposed as a nontoxic compound which specifically suppresses tubular secretion of penicillin. The present study is an attempt to evaluate its efficiency in routine clinical use in patients having normal kidney function, and in those suffering from various forms of renal disease.

A Comparison of the Pressor Activity of Desoxycorticosterone Acetate in Normotensive and Hypertensive Subjects. George A. Perera and (by invitation) David W. Blood, New York, N. Y.

Previous studies demonstrated that desoxycorticosterone acetate (DCA), administered to patients without hypertension or adrenal disease, may be associated with increases in blood pressure. Significant changes were apparent only after several weeks of drug injection.

Observations were made on ten normotensive subjects and twelve patients with uncomplicated hypertensive vascular disease maintained on a constant regimen and after a preliminary baseline period. The administration of DCA for one week (5 mg. subcutaneously twice daily) failed to alter the blood pressure significantly of any of the normotensive group. In contrast, definite elevation of systolic and diastolic readings took place in one to four days in the hypertensive individuals, the mean systolic rise being 24 mm. of mercury at the end of six days, the mean diastolic rise being 15 mm.

Alterations in weight, hematocrit, serum volume and electrolytes, urine volume and chlorides were comparable in both groups. Ballistocardiographic tracings were unchanged in one hypertensive subject following a marked rise in blood pressure after DCA.

Although prolonged administration of DCA may give rise to a gradual increase in blood pressure in normotensive subjects, an accelerated response to DCA may be found in hypertensive vascular disease.

Measurement of Renal Blood Flow and Glomerular Filtration During Variations in Blood Pressure Related to Changes in Emotional State and Life Situation. John B. Pfeiffer and Herbert S. Ripley, New York, N. Y. (Introduced by Harold G. Wolff).

Renal blood flow and glomerular filtration were ascertained by clearance measurements of para-aminohippuric acid and inulin respectively in ten human subjects with early hypertension. After a suitable control period in which subjects were reassured and made as comfortable as possible, topics of great significance in the life situation were abruptly brought up for discussion. In each instance the subject responded to the interview with a significant elevation of systolic and diastolic pressure but none gave evidence of fright or panic. Often their attitude was one of apparent calm and restraint during the period of elevated blood pressure. Evidence of renal arterial constriction was adduced in all cases during or after the interview. In four of the subjects a decrease of 20 percent or more in renal plasma flow occurred, one of these displaying also a significant decrease in glomerular filtration. In five of the subjects, despite elevation in systemic arterial pressure, no significant change in renal blood flow was found. In two of these an elevation of glomerular filtration occurred.

Subjects in whom lowering of blood pressure was induced by injection of tetraethylammonium chloride a significant increase in renal blood flow was noted. Similar lowering of blood pressure associated with increase in renal blood flow occurred when feelings of security were induced with the aid of sodium amytal.

The Excretion and Reabsorption of Bicarbonate by the Normal Human Kidney. Robert F. Pitts, Syracuse, N. Y. (Introduced by James A. Shannon).

The renal excretion and reabsorption of bicarbonate was studied in three normal subjects over a range of plasma concentration from 18 to 39 millimols per liter. Plasma bicarbonate was reduced by the ingestion of ammonium chloride, raised by the intravenous infusion of sodium bi-

carbonate. Blood was drawn from a cutaneous vein after heating the arm in water at 47° C.; both blood and urine were collected with precautions to prevent loss of carbon dioxide. The thiosulfate clearance was used as a measure of glomerular filtration rate. Carbon dioxide and pH measurements on plasma and urine permitted the calculation of bicarbonate contents and carbon dioxide partial pressures.

At plasma concentrations below 25 millimols per liter, the tubular reabsorption of bicarbonate was essentially complete. At plasma concentrations above 25 millimols per liter, the rate of excretion of bicarbonate progressively increased. There appears to be a limitation of the capacity of the tubules to reabsorb bicarbonate, the maximum quantity reabsorbed in the several subjects varying between 2.6 and 3.0 millimols per 100 cc. of glomerular filtrate.

The acidity of the urine varied between pH 4.48 and pH 7.80; the partial pressure of carbon dioxide in the urine varied from about 30 to nearly 200 mm. Hg. The more alkaline the urine, the higher was the partial pressure of carbon dioxide.

Hypoelectrolytemia in Peritonitis. S. RAPOPORT and (by invitation) I. A. SYLLM, N. NELSON, W. A. BRODSKY and K. DODD, Cincinnati, Ohio.

A decrease in the concentration of chloride and frequently of sodium has been described in tuberculosis, congestive heart failure, and in febrile diseases such as lobar pneumonia and typhoid fever.

In the present study, a lowering of the serum chloride and sodium, often accompanied by elevation of CO_2 content and pH, has been observed in children with peritonitis following rupture of the appendix. In some critically ill patients hypocalcemia was found. Clinically hyperpnea, a mild degree of edema, and decreased awareness of the surroundings were noted at the time of the appearance of the plasma changes. The disturbance of the electrolytes occurred within one or two days after the onset of the peritonitis and was self-limited in cases with a favorable outcome.

On an intake of 50—250 millimols of sodium chloride daily the urinary concentration of chloride and sodium was 20—60 millimols per liter; the hypoelectrolytemia persisted and retention of the sodium and chloride occurred.

The Pathogenesis of Hypercalcemia in Multiple Myeloma. ARNOLD J. RAWSON, Philadelphia, Pa. (Introduced by F. William Sunderman).

The calcium partition was studied in sera obtained from normal individuals and from patients with multiple myeloma. In normal sera the average value of calcium bound to albumin was found to be 0.95 (s.e. = 0.045) mg. of calcium per gm. of albumin, and that of calcium bound to globulin, 0.74 (s.e. = 0.033) ml. of calcium per gm. of globulin. In the sera of patients suffering from multiple myeloma the calcium binding property of albumin was increased to as much as six times the normal; the concentration of diffusible calcium and the calcium binding property of globulin in these sera remained within the normal range of values. Our studies indicate that when hypercalcemia is observed in patients with multiple myeloma, the increase is due to the calcium bound to protein. This increase in bound calcium is dependent upon two factors: (a) the increased calcium binding power of the serum albumin, and (b) the increased concentration of globulin, with approximately normal calcium binding property.

Renal Tubular Back-Diffusion Following Anuria. JULES REDISH, JOSEPH R. WEST and BETTY W. WHITEHEAD (by invitation) and HERBERT CHASIS, New York, N. Y.

A negative value for $T_{m_{pah}}$ was observed two and three and one half weeks following anuria in a patient with sulfathiazole intoxication. This negative T_m indicates not only that the tubular cells had lost their ability to excrete PAH, but that a portion of the PAH filtered by the glomeruli was escaping from the tubular urine by back-diffusion.

Observations extending from two to ninety-two weeks from the onset of anuria showed a pattern of glomerular and tubular recovery. Glomerular filtration was markedly reduced for three weeks following the onset of anuria and then returned abruptly to the normal range. Tubular excretory capacity was impaired for some weeks thereafter.

The data show the limitations of clearance measurements of filtration rate, renal blood flow and maximal tubular excretory capacity in the diseased kidney; and they suggest that a common mechanism, renal vasoconstriction with ischemia, may operate to produce glomerular and tubular changes during and after a period of anuria, whether the anuria is associated with diffuse glomerulonephritis, post-transfusion reaction, sulfathiazole intoxication, crush syndrome, shock or ureteral trauma.

Clinical and Experimental Observations on the Lability and Range of Blood Pressure in Essential Hypertension, with Special Reference to Physiologic Mechanisms and Significance of Various Pressor and Depressor Tests. MORTON F. REISER (by invitation) and EUGENE B. FERRIS, Cincinnati, Ohio.

Important in selecting the type of therapy for the patient with essential hypertension is a careful evaluation of the "neurogenic factor." As part of a comprehensive clinical, physiologic, and psychiatric study of essential hypertension, data pertinent to this problem has emerged. Comparative results in 107 pressor and depressor tests (1) performed on twenty-three patients indicate that extreme caution must attend their interpretation in the individual case.

Variations of blood pressure in response to emotional or environmental stimuli may equal or exceed those elicited by the usual pressor and depressor tests. The patient's response to different tests, or the same test repeated, may vary considerably, and there may be significant response to only a few of many procedures. There is no constant correlation between degree of reactivity to pressor tests and clinical lability.

In four patients with malignant hypertension, reductions in pressure to or very near normal occurred, three in re-

sponse to sympathetic block, one spontaneously. This together with other data (2) to be presented suggests that neurogenic factors play a significant contributing role even in this stage.

Preliminary studies utilizing the differential effects on blood pressure and cold pressor test of procedures which block neurogenic responses in different portions of the arteriolar bed have been helpful in leading toward a better understanding of the basic physiologic mechanisms involved and in explaining some of the apparent incongruities (3) of results which make selection of patients for sympathectomy so difficult.

(1) Test procedures used singly or in various combinations include: cold pressor; breath holding; postural changes; provocative interviewing; ammonia inhalation; hyperventilation plus carotid sinus massage; environmental manipulation; caudal, spinal, pentothal and gas-oxygen-ether anesthesia; and parenteral administration of the tetraethylammonium ion.

(2) In four instances we have observed the development of malignant hypertension and hypertensive encephalopathy in direct response to known environmental situations which, when appraised in the light of the psychiatric history, had specific psychological meaning for the patients.

(3) *E.G.*, in some patients who exhibit spontaneous downward variations in blood pressure to normal levels, spinal or caudal anesthesia may fail to produce such a satisfactory fall in pressure. When, in this circumstance, the tetraethylammonium ion, which effects a total autonomic block, is able to reproduce the fall to normal it seems probable that extra splanchnic arteriolar resistance may be playing an important role.

A Specific Cutaneous Test for the Immune Response to Herpetic Infection. Harry M. Rose and (by invitation) Eleanora Molloy, New York, N. Y.

Chick embryos inoculated by the yolk sac route with the virus of herpes simplex develop a high titer of virus in the chorioallantoic fluid. Antigens for cutaneous testing were prepared from this infected, undiluted fluid by inactivating the virus at 56° C. Such antigens contain little egg protein and provoke a minimal number of non-specific dermal reactions.

Cutaneous tests were performed on 44 patients with herpes antigens and with control antigens prepared from normal chorioallantoic fluid. The blood of each patient was examined for neutralizing antibody to herpes virus by the method of chorioallantoic membrane inoculation with serum-virus mixtures.

Twenty-nine patients whose sera contained antibody gave positive cutaneous tests with herpes antigen and negative tests with control antigen. Thirteen patients, mostly children, whose sera were devoid of antibody gave negative cutaneous tests with both antigens. In one patient the cutaneous tests were negative although the serum contained antibody. In another patient the tests could not be interpreted because of marked sensitivity to egg protein.

There appears to be a high degree of correlation between positive cutaneous reactions to herpes antigens, as herein described, and the presence of circulating antibody to herpes virus.

Recognition of Metastatic Neoplasm in Bone Marrow. R. W. Rundles, Durham, N. C. (Introduced by D. T. Smith).

Invasion of the bone marrow by neoplasm may produce refractory anemias, sometimes with immature granulocytes or nucleated red blood cells in the circulating blood, hemolytic anemias, thrombopenic purpura, as well as diffuse bone pain, destructive skeletal lesions, etc. Diagnosis may be extremely difficult when the primary tumor escapes recognition or if involved tissue is not accessible for biopsy.

The possibility of recognizing tumor implants in bone marrow was studied in 40 patients having malignancies with local spread or distant metastases. Marrow was obtained from the ribs or sternum by combined aspiration and needle trephine preferably in areas of increased bone tenderness. Spread films were stained by Wright's stain A positive diagnosis of metastatic neoplasm was made in 14 cases. The malignant cells occurred singly, in sheets, and in clumps among other bone marrow elements. The presence of neoplasm in the marrow was confirmed by sections of tissue obtained by trephine, biopsy and necropsy.

Neoplastic implants in the bone marrow were found in patients before definite roentgen, peripheral blood, or blood chemical abnormalities appeared. Serial biopsies were readily obtained and were used to follow the response of prostatic metastases to orchidectomy and estrogenic therapy, the response of breast cancer to androgenic hormones, and other malignancies treated with nitrogen mustard compounds or radiation therapy.

Pitressin Test of Coronary Insufficiency. Arthur Ruskin, Galveston, Texas (Introduced by George M. Decherd).

Pitressin, a well-known experimental coronary vasoconstrictor, was used in human subjects both intramuscularly (2 cc. or 40 pressor units) and intravenously (0.75 cc. in subjects of average weight) for the electrocardiographic demonstration of coronary insufficiency. The not infrequent untoward symptoms—nausea, vomiting, abdominal discomfort—were easily controlled. In normal individuals EKG changes were limited to bradycardia, ectopic beats and slight S–T segment depressions.

In cases of definite clinical angina pectoris positive tests were found in 8 of 12 cases. The number of positive Master two-step tests in these 12 patients was 7. Our electrocardiographic criteria of coronary insufficiency were fairly rigid: S–T depressions totalling 3 mm. or more in the 4 commonly used leads, or T_1, T_2, or T_4 changes from positive to flat, diphasic or negative T waves. All 10 cases of suspected pseudo-angina, with indefinite clinical features, had negative pitressin and Master tests. Patients with abnormal EKG's prior to the test were found to be subject to the same criteria in both groups as were those with normal control tracings.

In view of the close parallelism between the effects of exercise and pitressin in individuals with and without clinical angina pectoris, pitressin may be used by cardiologists to confirm the clinical suspicion of coronary pain. The above results form a further link in the evidence for both coronary spasm and relative myocardial ischemia as theoretical bases for clinical anginal pain.

Microchemical Studies of Human Urinary Estrogens. W. T. SALTER and (by invitation) M. JANE OESTERLING, New Haven, Conn.

Studies of estrogen excretion in various types of patient and normal controls have been conducted on 6-hour to 12-hour samples of urine. The normal adult human male excretes only about 10 micrograms of estrogenic substances daily. Near menstruation, some women approach closely the male value. In the intermenstruum, however, female values rise steadily toward the time of ovulation. At this crisis, the peak of excretion temporarily may reach 90 micrograms. In certain sterile women, the male level may prevail continuously. In other sterile women, the estrogen level may be normal, but overshadowed by high "androgens." Therefore it is useful to know the ratio of antithetical steroids (estrogens vs. 17-ketosteroids) in the identical 6-hour sample of urine, as well as the absolute levels of either. The "antithetical ratio" of normal males is usually less than 1.0. In normal females, it is usually above 2.0 and at ovulation may reach 9. In female adrenal virilism and in Cushing's syndrome the ratio is usually in the male range. In female pseudohermaphroditism, however, the high "androgen" excretion may be balanced by a high estrogen value. In prostatic disease, high ratios are found. Thus studies of steroid balance may be useful in precise diagnosis.

Nitrogen Metabolism and Nutritional Studies in Chronically Ill (Metabolically "Debilitated"), Undernourished Patients. VICTOR SCHENKER and L. G. JOHNSON (by invitation), and J. S. L. BROWNE, Montreal, Canada.

The nitrogen metabolism, nutrition, and clinical course have been followed in a series of hospital patients with various chronic diseases (pulmonary tuberculosis, rheumatoid arthritis, bronchiectasis, peptic ulcer), and with varying degrees of weight loss. Representative experimental results are presented.

All subjects were placed on controlled dietary regimes over certain periods of time. Experimental diets varied as to total content of protein and calories (Prot. 100–200 gm./ day; Cals. 2,500–3,500/ day).

On these food intakes all subjects gained weight; the majority showed general improvement clinically with increased strength and feeling of well-being.

All showed abnormally low rate of urinary nitrogen excretion (with a consequent high nitrogen retention—unattributable to raised blood N.P.N.), which has been shown to characterize the metabolically "debilitated" individual. In many instances, however, nitrogen retention continued at the same rate even after considerable amounts had been stored over prolonged periods of time, and increase in body weight ceased. Further, in a number of cases, the total nitrogen retained, calculated as "tissue-protein," was found to be greatly in excess of the actual total gain in weight. Factors which may be responsible for these anomalies are discussed.

Radioiodine Excretion in the Gastric Juice and Saliva of Man. LEON SCHIFF and (by invitation) CHARLES D. STEVENS, HAROLD H. STEINBERG, CARL W. KUMPE and PAUL STEWART, Cincinnati, Ohio.

The excretion of radioiodine in the gastric juice and in the saliva of 35 individuals was followed at ten-minute intervals for two hours after the intravenous administration of 0.01 *mM* (1.5 mg.) of NaI and 4 microcuries of radioiodine per kgm. of body weight. Plasma levels of 0.04 to 0.08 *mM* of iodine per liter 15 minutes after the injection declined to 0.01 to 0.02 *mM* by 1½ hours after injection.

The concentration of radioiodine in gastric juice following the injection usually rose to 20 to 40 times the plasma concentration. The concentration in the saliva also rose to similar levels, frequently exceeding the concentration in the gastric juice.

The concentration of radioiodine in both the gastric juice and the saliva showed great variability from individual to individual, as well as upon repeated tests on a single individual. The variability in radioiodine excretion in the gastric juice was not related to the pH of the juice, nor to its volume, nor to the amount of visible mucus in it.

No definite relation has been observed between any gastric disorders and the excretion of radioiodine in the gastric juice.

*The Metabolism of Americium (Element 95) in the Rat.** K. SCOTT, D. AXELROD, and H. COPP (by invitation), and J. G. HAMILTON, Berkeley, Calif.

Solutions of $Am(NO_3)_3$ were administered to 200-gram rats by intramuscular injection and by stomach tube. The stomach tube series were sacrificed at 4 days, and the animals which received the americium by intramuscular injection were sacrificed at 1, 4, 16, 64, and 256 days. The absorption from the digestive tract was too small to be detected, indicating that less than 0.01 per cent of the administered dose was absorbed. The americium that was absorbed following intramuscular injection was accumulated primarily in the liver and skeleton. The initial accumulation in the liver represented nearly 65 per cent of the total amount absorbed from the site of intramuscular injection. The corresponding value of americium deposition in the skeleton was approximately 25 per cent. Within 16 days over two-thirds of the americium had left the liver and by 64 days less than 5 per cent remained in that organ. The content of americium in the skeleton remained constant up to and including the 256-day interval; evidently it becomes extremely firmly fixed in bone. Excretion was chiefly by way of the digestive tract and was roughly equivalent to the rate and degree of loss from the liver. Radio-auto-

* This work was performed under Contract W-No. 7405-eng-48-A for The Manhattan Project.

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